Optimisation of thermal processing of fresh farmed cod

Optimalisering av varmebehandling av fersk oppdrettstorsk

Philosophiae Doctor (PhD) Thesis

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Preface

After having pursued a PhD position for some time, Torstein Skåra, who was research manager at Norconserv AS (now Nofima) took initiative to a research proposal to the Research Council of Norway who funded the project called ConCod (158929/I30). This gave me the privilege to combine my work with a PhD-study.

Heat preservation of fish is a challenging task and this was clearly expressed by Jarle Vidvei at Fjordkjøkken AS: - The heat treatment makes the fish dry and though; how can we avoid that? From this point I started on a twisted travel to answer that question, starting in Stavanger working shoulder to shoulder with Merete Lund Østby. Dr. Ragni Ofstad at Matforsk (now Nofima) gave me a good overview based on her own work on farmed cod. Next destination was Katholieke Universiteit Leuven where I was supervised by Professor Marc Hendrickx and his co-worker dr.ir. Iesel van der Plancken at the Laboratory of Food Technology. During the first year it was concluded that a new method for determination of water holding capacity was needed and so a method and new equipment was invented. The invention was promoted by Sven Tore Sivertsen at Prekubator and a licese agreement was made with Andreas Hettic GmbH, Tuttlingen.

Another destination visited was Tromsø where I was supported by Gunn Berit Olsson, Hilde Herland, Margrete Essaiassen, Heidi Nilsen and others at Fiskeriforskning (now Nofima) and Silje Kristoffersen, Ragnvald Olsen and Edel Elvevoll at the University of Tromsø. At the Danish Technical University in Copenhagen I was supervised by Flemming Jessen and his team during an intensive and highly relevant course on fish muscle biochemistry.

During all these twists Stavanger has been the home base. My colleagues at Nofima have created an enabling environment and some have to be especially mentioned; Åsvald Vågane has solved several practical obstacles, Jan Thomas Rosnes has thought me some microbiology, Bjørn Tore Rotabakk has served with tips and hints, Karin Tranøy and Laila Budal has assisted in the analysis and Sissel Johnsen has been working close together with me and generously sharing her knowledge.

I want to express my sincere gratitude to all those mentioned above and in particular my supervisors associate professor Odd-Ivar Lekang at UMB and Morten Sivertsvik at Nofima, without whom I would never got back on the track to accomplish a PhD.

Thank you to all family members who have been very supportive, especially my parents Paal and Karen and my parents in law, Unni and Bjørn. It was my intention to perform this work without compromising a normal family life; nevertheless I could never have done this without the love and encourage from my wife Stine, our daughter Marte and our son Pål who was born into this. Thank you very much for your patience!

Stavanger, 06.02.2011

Dagbjørn Skipnes

Abstract

Heat treatment of cold water species is challenging due to quality changes occurring at low temperatures relative to most other foodstuffs. One of the characteristic challenges is the melting of connective tissue already below 40°C, causing disruption of the myotoma i.e. flaking of the fish muscle. One of the most heat sensitive fish species is Atlantic cod. Due to the relatively low fat content compared to other fish species, Atlantic cod easily loses its juiciness when the heat load causes cook loss or loss of ability to bind the inherent water (water holding capacity). In addition a tough texture is experienced after heat treatment at high temperatures. Farmed cod is known to lose its water holding capacity (WHC) earlier than wild cod during storage in raw condition. On the other hand, the market demand for Atlantic cod is high and farming is promising for regularity in delivery of cod and makes further processing profitable. The combination of the potentials for farmed cod and the challenges in heat processing has made the farmed Atlantic cod an interesting object to study.

In this work, the quality of vacuum packaged, heat processed cod loins has been studied in order to find optimal combinations of processing time and temperature. A sample cup has been developed for rapid and homogenous heating and cooling of a fish sample with the possibility to measure cook loss, water holding capacity and texture without removing the sample from the cup. The sample cup and associated method have been used to characterise water holding capacity of the fish muscle at 156 combinations of temperature and processing time. The water holding capacity showed to decrease rapidly as soon as reaching the denaturation temperature of the proteins, but the correlation to temperature and processing time was complex and non-linear. The stepwise and partially overlapping denaturation of protein groups during heating is the major explanation for this behaviour and therefore the enthalpy changes during protein denaturation was investigated. The energy required for denaturing the protein groups has been scanned at a constant heating rate of

10°C/min. Five partially overlapping peaks each concentrated around a maximum peak temperature was observed, each representing the energy required for denaturation of a protein or a group of proteins. The denaturation of each of these five protein groups has an effect on the cod muscle. The peak maximum temperature for myosin was found at 44.1°C. However, myosin denaturation starts at about 28°C and continues to about 50°C and during this process the myosin chains will contract, split and form a new and more open structure with reduced ability to bind water. From 50°C to 72°C sarcoplasmic proteins are denatured, and the muscle is shrinking and partially closing the capillaries that were opened. Thus, the water holding capacity is increasing again but this development is reversed by denaturation of actin which starts already at about 58°C. At this temperature the denaturation of actin is a slow process and even at 68°C it will take about 13 min to denature 90% of the present actin and the peak maximum is reached at 76.1°C. One interesting observation is the rapid increase in cook loss when the water holding capacity again starts to decrease. No further protein denaturation was observed in the temperature range from 88°C to 120°C by the calorimeter, but still there was an increase in cook loss. General physical and thermodynamic inherent orderliness which applies to all porous materials is expected to at least partly be the reason for this cook loss and it should therefore be possible to calculate it if the gradients of temperature, pressure and concentration of liquid can be determined over the cross section of the cod loin (the thick part of the fillet).

In general, the fish texture gets harder with increasing temperature, but as discussed for water holding capacity, there are no simple and obvious correlations to processing time and temperature. The surface of the cod is changing from opaque to white when heated to about 50°C, but after this transformation changes in colour can hardly be observed.

The required heat load on a convenience cod product is determined by the desired shelf life and microbial constraints. These constraints must be known with accuracy to optimise the heat treatment as well as the temperature distribution over the whole volume of the cod loin. Temperature measurements in the core of the loin may be sufficient

when using conventional heating methods, like autoclaves. This is either impractical or insufficient for a number of heating processes, e.g. continuous heating in tunnels and microwave heating. Even in some conventional heating processes, e.g. convection ovens, the ambient temperature distribution and temperature controlling are so poor that the cold spot in the fish cannot be determined. In such cases the inactivation of microbes and enzymes could rather be evaluated by inherent biological markers. One such promising biological marker described in literature was the residual enzymatic activity of acid phosphatase (ACP). Extracts of raw cod muscle was heat treated by several time and temperature combinations. A clear correlation between residual ACP activity and heat load was found. ACP levels also showed to be insignificantly dependent on gender or fish size and seasonal variations were small. The work was continued by heat treatment of fish muscle and subsequent extraction of aliquots. In the range 56°C to 68°C residual ACP activity showed to have some potential as a marker for inactivation of *Listeria monocytogenes*.

The results discussed above may be used directly for optimisation of a thermal process. For instance, the ranges of time and temperature combinations that may give high water holding capacity, low cook loss and safe inactivation of *L. monocytogenes* have been identified. Further work should focus on how to combine this knowledge with heat transfer model of the fish portion that is able to predict the temperature development at any location of the fish. Such a model is already available as an example. The observations of quality changes presented in this thesis have been made over a wide range of time and temperature combinations. It should therefore be possible to model and optimise most heat treatment processes of interest to food companies processing vacuum packaged cod loins by combining the observations in a numerical model.

Sammendrag

Varmebehandling av kaldtvannsfisk er utfordrende fordi uønskede kvalitetsendringer kan oppstå ved lavere temperaturer enn mange andre matvarer. Ett av disse særtrekkene er at bindeproteinet kollagen selv ved temperaturer under 40 °C kan denatureres slik at myotomene (skivene) i fiskemuskelen faller fra hverandre. Et av fiskeslagene som lett faller fra hverandre ved varmebehandling er torsk. Med sitt lave fettinnhold i muskelen kan Atlantisk torsk i tillegg lett oppleves som tørr dersom varmebehandlingen medfører stort væsketap eller tap i evnen til å binde vann i tillegg til at muskelen blir hard ved høyere temperaturer. Flere studier har vist at oppdrettstorsk kan være særlig utsatt for tap av vannbindingsevne i rå tilstand. Samtidig er torsk en etterspurt matvare og oppdrettsfisk åpner for en regularitet i leveransene til markedene som muliggjør økt foredlingsgrad. Kombinasjonen av oppdrettstorskens potensial og at den er mer utfordrende enn andre fiskeslag har gjort den til et spennende studieobjekt.

I dette arbeidet har kvaliteten på torskefilet (loin) som er varmebehandlet i en vakuumpakke blitt studert i den hensikt å finne en optimal kombinasjon av varmebehandlingstid og -temperatur. Det har blitt utviklet en prøvekopp egnet for hurtig og homogen oppvarming, varmholding ved konstant temperatur og nedkjøling av en liten fiskeprøve. Uten å fjerne prøven fra koppen er det mulig å måle koketap, vannbindingsevne og tekstur. Prøvekoppen og tilhørende målemetoder er brukt til å karakterisere vannbindingsevnen i fiskemuskelen ved 156 kombinasjoner av temperatur og varmebehandlingstid. Disse målingene har vist at vannbindingsevnen tapes raskt straks temperaturen blir tilstrekkelig høy for denaturering av proteiner. Koketap og vannbindingsevne er imidlertid parametre som har vist seg og ha en komplisert og ikke-lineær sammenheng med varmebehandlingstid og –temperatur.

Forklaringen på disse fenomenene er i vesentlig grad knyttet til at denatureringen av ulike proteingrupper skjer stegvis og delvis overlappende under oppvarming, og derfor ble energiomsetningen ved proteindenaturering undersøkt. Energimengdene som medgår til

proteindenaturering er blitt målt under konstant oppvarming av fiskekjøttet og det er påvist at energiforbruket er konsentrert i fem, delvis overlappende, topper rundt hver sin maksimumstemperatur. Hver topp representerer energien som medgår til denaturering av et protein eller gruppe proteiner. Proteindenatureringen som foregår i disse fem stegene har ulike effekter på fiskemuskelen. Når temperturen når 44,1 °C er denaturering av myosin på sitt maksimale og proteinkjedene vil trekke seg sammen og aggregere med hverandre slik at det dannes en ny og åpnere struktur i fiskemuskelen. Samtidig vil de spaltede proteinkjedene klumpe seg sammen. Vann som var immobilisert i myofibrillene presses dermed ut i det ekstracellullære området. Dette er bakgrunnen for at vannbindingsevnen faller vesentlig i temperaturområdet fra 28 °C til 50 °C. Fra 50 °C og oppover denatureres sarkoplasmiske proteiner og etter hvert som muskelen krymper vil de kapillare kanalene som åpnet seg bli lukket igjen slik at fallet i vannbindingsevne avtar og faktisk reverseres i området 50 °C til 72 °C. Denne effekten vil imidlertid bli reversert når aktin denatureres. Aktindenatureringen starter allerede rundt 58 °C men krever lang tid ved så lav temperatur og selv ved 68 °C vil det ta omkring 13 min å denaturere 90% av tilstedeværende aktin og når først sitt maksimale ved 76,1 °C. En interessant observasjon er at koketapet tiltar kraftig når vannbindingsevnen faller igjen. Denne utviklingen fortsetter når temperaturen økes ytterligere selv om ytterligere proteindenaturering ikke kan observeres kalorimetrisk i området 88 °C til 120 °C. Det antas derfor at væskeutskillingen i tillegg til effektene av proteindenaturering følger allmenne termodynamiske lovmessigheter for porøse materialer. Derfor kan trolig væsketapet beregnes ut fra gradienter i trykk, temperatur og konsentrasjon av væske gjennom tverrsnittet av torskefileten.

Generelt blir fisken hardere ved kraftigere varmebehandling, men i likhet med vannbindingsevnen skjer ikke endringene konstant verken i forhold til varmebehandlingstid eller -temperatur. Fargen endres vesentlig ved lave temperaturer og fileten går hurtig fra delvis gjennomskinnelig til hvit, men fra 50 °C og oppover er endringene knapt målbare.

Holdbarhet og sikkerhet utgjør rammebetingelsene for påkrevd varmebelastning for ferdigretter av torsk. For å kunne optimalisere

varmebehandlingen må disse rammebetingelsene bestemmes med best mulig nøyaktighet. I tillegg er det en forutsetning å kjenne temperaturforløpet over hele volumet av loinen (den tykkeste delen av fileten). Ved konvensjonelle varmebehandlingsmetoder som vannbad og autoklaver der produktet er stillestående under varmebehandlingen, kan temperaturmålinger utføres i enkelte punkter (fortrinnsvis i produktets kaldeste punkt) under oppvarmingen. Dette er imidlertid upraktisk i en rekke varmebehandlingssytemer, som ved kontinuerlig varmebehanding (koketunneler, hydrostatiske autoklaver) eller mikrobølgeoppvarming. I kokeskap vil også store variasjoner ulike steder i skapet, dårlig regulering og dårlig repeterbarhet gjøre det problematisk å utføre representative målinger. I disse situasjonene kan det være avgjørende å kunne evaluere varmebelastningen på enzymer og bakterier etter at varmebehandlingen er gjennomført ved hjelp av en iboende biologisk indikator. I litteraturen ble det funnet en slik indikator som var regnet som lovende, nemlig resterende enzymaktivitet fra sur fosfatase (ACP). Ekstrakt fra prøver av rå torsk ble utvunnet og varmebehandlet med ulike kombinasjoner av tid og temperatur. Det ble funnet en entydig sammenheng mellom varmebelastningen og resterende aktivitet av ACP i hver av prøvene. Innholdet av ACP viste seg å være ubetydelig påvirket av variabler som størrelse og kjønn på fisken og sesongvariasjonene var små. Arbeidet ble ført videre med varmebehandling av prøver av fisk og påfølgende ekstrahering og analyse av ACP. Det viste seg at innenfor temperaturområdet 56 °C til 68 °C kan ACP i en viss utstrekning brukes som en indikator for inaktivering av *Listeria monocytogenes*.

Resultatene som er beskrevet ovenfor kan benyttes direkte til optimalisering av varmebehandlingen. For eksempel er det vist hvilke kombinasjoner av tid og temperatur som gir høy vannbindingsevne og lavt koketap samtidig som *L. monocytogenes* inaktiveres dersom temperaturforløpet i fisken under varmebehandling og kjøling er kjent. Derfor vil det være tjenlig å videreføre arbeidet som er gjort ved å integrere resultatene med en numerisk modell for varmetransport. Siden målingene som er gjort dekker de fleste aktuelle kombinasjoner av varmebehandlingstid og –temperatur kan matindustrien bruke en slik modell til å optimalisere de fleste varmebehandlingsprosesser tilpasset vakuumpakkede torskeloins.

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These publications are referred to by their roman letters in the following chapters.

Introduction

From a thermodynamic point of view, foods are unstable in the sense that they tend to change from a state of low entropy and high enthalpy to a state of low enthalpy and high entropy. In food technology we counteract this thermodynamic instability by several means, one of them being thermal processing.

Preservation by heat remains one of the major methods for extending the shelf life of packaged fish. By doing the heat preservation after hermetically sealed packaging, microorganisms are prevented from contaminating the processed food. In-pack thermally processed fish products have been produced for almost two centuries. While Nicolas Appert started the first cannery for meats and vegetables in 1803 (Appert, 1810), the first production of canned sardines took place in France in 1830. As one of the results of the ongoing globalization process, the exported volume of canned seafood products increased from 11 805 000 tons in 1998 to 17 106 000 tonnes in 2008 (FAO, 2009).

Healthy eating trends have given rise to increased demand for fish and seafood which is acknowledged to offer many health benefits. In addition, heart health has become a major concern among modern consumers as heart disease being responsible for a large number of deaths in the developed world. The benefits of fish oil over animal fats are widely recognised among consumers and this has also led to a shift away from read meat towards white meat and more importantly fish and fish products. The health aspects of eating seafood have primarily been linked to marine lipids (Larsen et al., 2007) and marine Ω-3 polyunsaturated fatty acids (PUFA) are associated with reduced risk of coronary heart disease (Schmidt et al., 2006).

It has been observed a moral attitude amongst consumers in northern European countries against ready meals (Olsen et al., 2010), which could be expected to be less important for seafood ready meals. In spite of this, fish is underrepresented among the ready meals.

However, the ready meals market in western Europe was worth Euro $28.1*10^9$ in 2009 and is steadily growing (Food For Thought, 2011). The most important factor driving growth in the ready meals market is the increasing demand for convenience. Many consumers do not like to cook fish themselves, even if they like the fish and believe it is a nutritional alternative to meat products. Several reasons may be mentioned for this and some of them are the smell during cooking, bones in the fish and that the fish sticks to the pan. Another problem may be the relatively short shelf life of fresh fish, which means it has to be cooked shortly after purchase.

Thermal processing can be subdivided into several more or less overlapping groups, based on temperature regime, method or equipment for thermal processing, fish species, packaging method or the microbial target of the process.

Sterilisation is the classical method. The products are undergoing a process aiming for inactivation of all pathogenic bacteria and their spores. The temperature regime during processing may vary from 110°C to 135°C. For low acid foods (pH > 4.5) the process is aimed to inactivate the spores of *Clostridium botulinum* type A. This is sometimes referred to as commercial sterility, as some spore forming non-pathogenic strains may survive this heat load. Other sub groups of sterilisation are also in use but not presented here.

Pasteurisation is intended to inactivate vegetative cells but is not intended to inactivate the spores of all pathogenic bacteria. The term is often related to the heat treatment of acid foods or refrigerated foods where growth of surviving spores is prevented by a pH below 4.5, a low temperature or by other means. A variant of pasteurisation is Sous Vide processing, i.e. mild thermal processing of vacuum packaged products.

Minimally processed convenience foods are a growing segment in the European marketplace. Fish based products are underrepresented among these foods, due to a number of unsolved problems. The products from the traditional fish processing industry have an unpredictable quality and suitability for minimal processing due to seasonal variations, variations in freshness and handling between

catch and filleting, as well as differences in the functional properties depending on the raw material history, e.g. fresh versus frozen raw material.

Farming of was until recently a fast growing industry and received much attention e.g. (Jobling, 1988; Tilseth, 1990; Puvanendran & Brown, 1999; Morais et al., 2001; Hemre et al., 2003; Lauritzsen et al., 2004a; Lauritzsen et al., 2004b; Stien et al., 2005; Kristoffersen et al., 2006; Forde-Skjaervik et al., 2006; Larsen et al., 2008; Hagen & Solberg, 2010; Bjornevik & Solbakken, 2010). After the first trials in Norway in the early eighties, intensive culture of cod has now been successful since 1996, and the commercial production has increased drastically since 2000 (Brown et al., 2003). Total export of farmed cod from Norway was 11 087 tons in 2006 (for compassion the export of farmed Atlantic salmon was 626 000 tons in 2006) (http://www.ssb.no/fiskeri_havbruk/) and increased to 20 683 tons in 2009 (Norwegian Directorate of Fisheries, 2010). However, farming of cod has not yet been profitable. One of the possible measures to meet this challenge is development of value added products.

In the fish processing industry, farmed and wild cod of different origins are exposed to a number of handling procedures. Commercial thermal processing of white fish species has not been common practice, and scientific data are scarce. Farmed cod is more suitable for minimal processing than wild fish, due to a more consistent and predictable quality and bacterial load (Herland et al., 2010). The use of intermediate processing steps on one hand and an optimised thermal process on the other should prove to be a good basis for successful combinations.

Optimisation of thermal processing with respect to quality and safety is only possible if we are able to quantify the kinetics of microbial inactivation and quantify quality retention. While thermal inactivation kinetics of many pathogen microorganisms is well known, information on quality changes during thermal processing often is lacking. Simple elementary chemical reactions can be well described, but interactions in the food matrix and individual biological variation are two complicating factors (van Boekel, 2008).

Objective of the study and organisation of the work

The present work focuses on quantification of quality changes in farmed cod during heating. It has been the aim to combine the findings on quality changes with collected information on microbiological constraints and physical characteristics of the fish to optimise the time and temperature combination used for pasteurisation of vacuum packaged convenience products.

This thesis is organised in four chapters. The first chapter is presenting **Background** on thermal processing and its effects on farmed cod. The second chapter is presenting the **Main results and discussion** of the present work. This chapter is organised like a toolbox for optimisation of thermal processing as illustrated in Figure 1. The third chapter is presenting the main **Conclusions** of the work. The fourth and last chapter is presenting a suggested path for **Further studies**. This last chapter includes an example on the building of a heat transfer model and an outline on how the main results can be combined with the heat transfer model for optimising a thermal process.

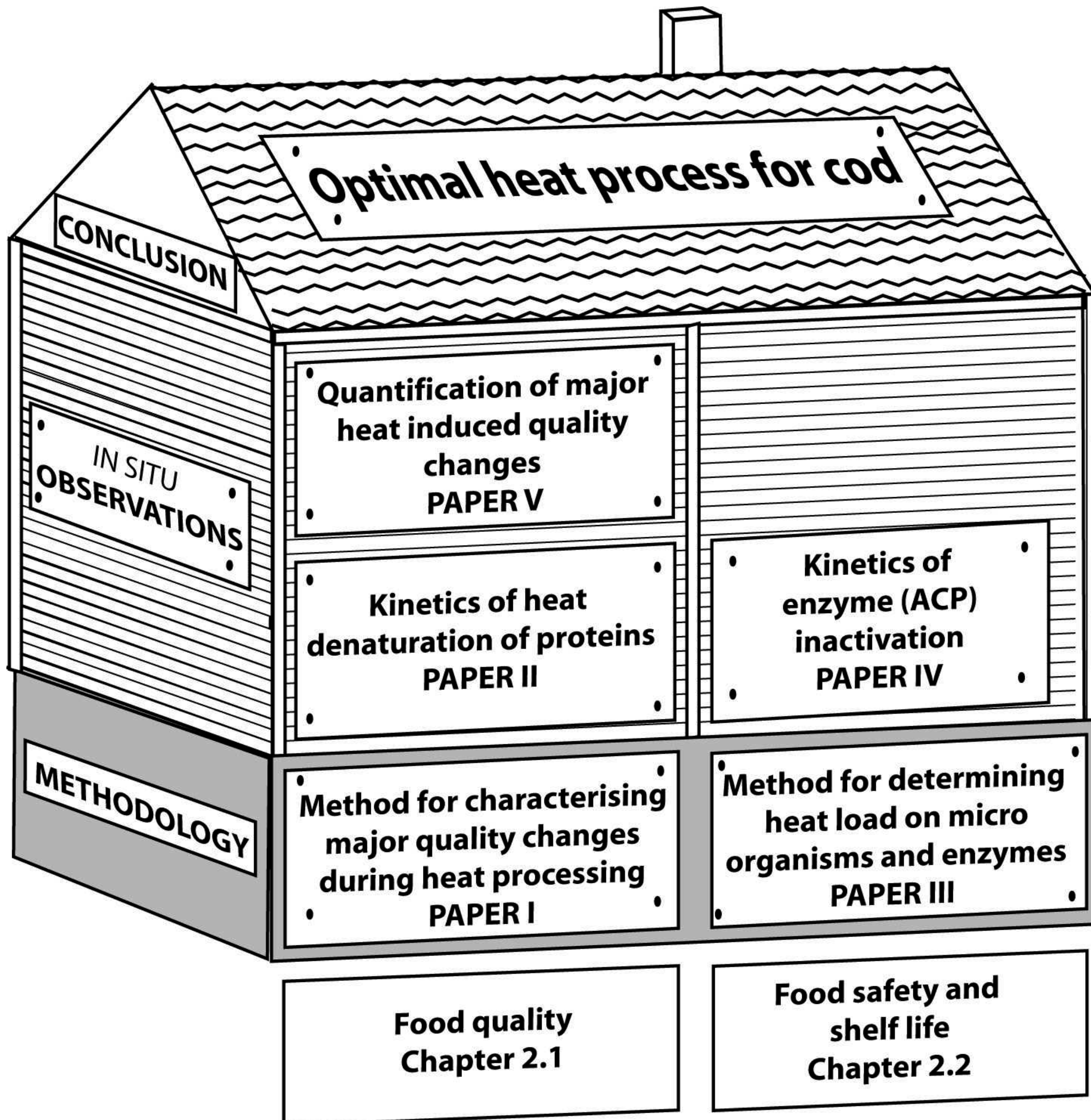

Figure 1. Organisation of the main results and discussion as a toolbox for finding optimal heat processes for cod

1. Background; effects of thermal processing on cod

The objective of this chapter is to summarise the current knowledge on mild thermal processing of cod and the specific challenges for minimally processed farmed cod in particular. A general overview on optimisation including canning of fish can be found in Skipnes & Hendrickx (2008).

1.1. Basic theory on thermal processing

One of the main reasons for thermal processing of fish products is to inactivate microorganisms. To determine whether the heat inactivation is sufficient or not, several methods may be used and the most widespread method is to determine lethality from recorded temperature. These methods were originally developed for sterilisation processes but later used for pasteurisation processes.

The original death kinetics work often quoted is that of Esty and Meyer (1922), who investigated the death kinetics of *Bacillus botulinus* (then name for *C. botulinum*). In this work heat inactivation showed to have be almost log-linear. In general, such behaviour may be described by a first order reaction, equation 1, and plotted as shown in figure 2.

$$\frac{dN}{dt} = -kN \qquad (1)$$

where N is the number of microorganisms at time t and k the inactivation rate constant (min^{-1}). In case of isothermal experiments (i.e. rate constant k not varying with time), equation 1 can be integrated, obtaining equation 2, where N_0 is the initial number of microorganisms (at zero minutes of the isothermal treatment):

$$N = N_0 \cdot e^{(-kt)} \qquad (2)$$

In the area of food science and technology, it is common to characterise first-order reactions using the Thermal Death Time concept. The decimal reduction time (D-value) is the time, at a given constant temperature, needed for a 90% reduction of the microorganism. For a first-order reaction, D-values and rate constants are inversely related, equation 3.

$$D = \frac{\ln(10)}{k} \qquad (3)$$

Substitution of equation 3 into equation 2 yields an alternative equation for a first order reaction, equation 4.

$$N = N_0 \cdot 10^{-\frac{t}{D}} \qquad (4)$$

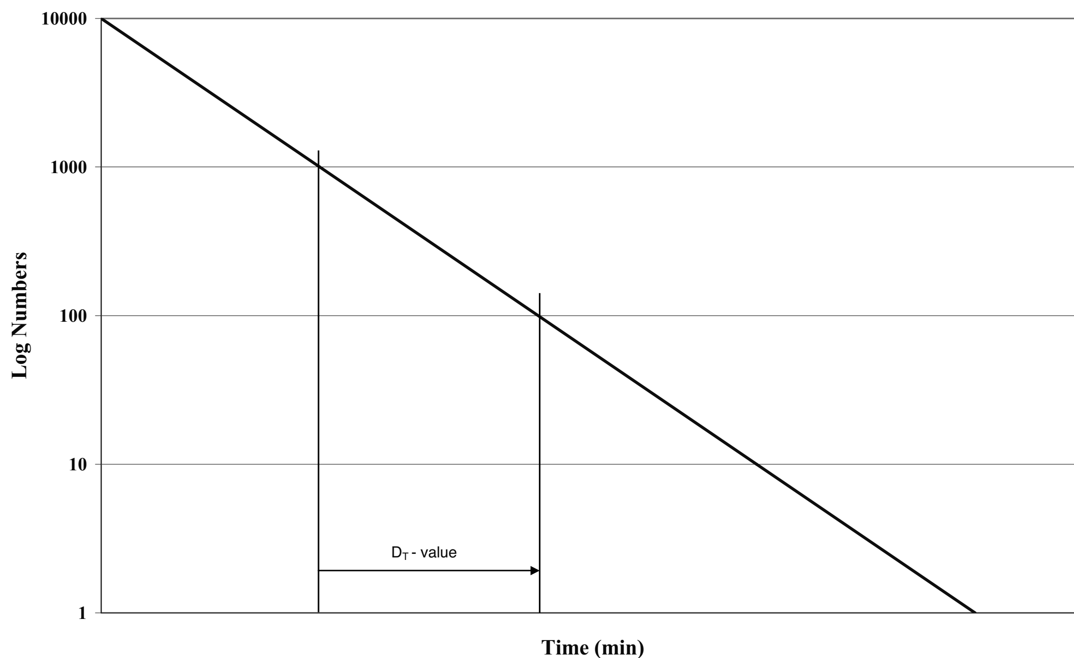

Figure 2. Logarithmic survivor curve showing the calculation of decimal reduction time (D_T-value), which is the time required to decrease the number of a specific microorganism by a factor of ten at a specific temperature T.

In the Thermal Death Time model, the temperature dependence of the D-value is given by the z-value, equation 5. The z-value equals the temperature increase necessary to obtain a tenfold decrease of the D-value.

$$D = D_{ref} 10^{\frac{T_{ref} - T}{z}} \qquad (5)$$

where D_{ref} is the decimal reduction time at reference temperature (T_{ref}), and z the z-value.

For practical use a pasteurisation value, P (or F for sterilisation), based on the kinetic parameters of heat inactivation is commonly in use and can be calculated as shown in equation 6.

$$P_{T_{ref}}^{z} = \int_0^t 10^{\frac{T_c(t) - T_{ref}}{z}} dt \qquad (6)$$

Common pasteurisation treatments are designed for a 6-log inactivation; for example, if the initial loading of *L. monocytogenes* is 10^3 /g then a 6-log process will reduce this to 10^{-3} /g. This number of surviving bacteria is best described as a probability of a bacteria surviving the process rather than an absolute number. The required P-value is shown in equation 7. The heating time, t, needed at constant temperature in order to obtain a 6-log inactivation is therefore equal to 6 times the D-value.

$$P_T = D_T \cdot \log\left(\frac{N_0}{N}\right) = D_T \cdot \log\left(\frac{10^3}{10^{-3}}\right) = D_T \cdot 6 \qquad (7)$$

Analogue to the pasteurisation process, a sterilisation process where 12-log reductions are required, the target F-value for *C. botulinum* spores with D-value of 0.21 min (UK Department of Health, 1994) at 121.1°C is 2.52 min. This value is rounded up to F_0 3 and is a well known criterion for a safe sterilisation process.

The basics of mathematical methods for calculation of a sterilisation process were developed in the 1920'ies. Ball's mathematical method,

after C. Olin Ball (1923), was published and has been refined several times. Other mathematical methods have been issued later and also adapted to pasteurisation processes, like the Stumbo calculation method (Stumbo, 1973). Modified versions of these methods are today found in computer programs side by side with numerical simulation. One example of this is CTemp (Tucker & Holdsworth, 1991; Tucker et al., 1996) issued by Campden BRI. Among the numerous publications in this field, (Pflug, 1988) presented several of the most central papers up to 1978. A review of the mathematical methods has been presented by (Stoforos et al., 1997) and there are also several publications on how to bring these methods to the next step: on-line control of autoclaves based on thermal inactivation modelling (Teixeira et al., 1999).

1. 2. The cod

Cod is a good source of protein, and the distribution of essential amino acids is close to the composition of human milk. Cod also contains a significant amount of vitamin B12 and selenium. Both the nutritional aspect and the sensory properties of cod have resulted in a high market demand.

The properties of farmed and wild Atlantic cod (*Gadus morhua*) vary with factors including fishing grounds, seasonal variation, fishing gears, feed, environment and post harvest treatment. Through breeding programmes and controlled farming conditions, several factors, like body size, growth rate and sexual maturation can be manipulated. A farmed cod reaches a weight of 3 kg two years after hatch, while wild Atlantic cod reaches the same weight after three to five years (Otterå & Akse, 1992). Fast growth affects the structure and texture of the muscle.

The macro structure of the fish fillets is shown in figure 3. The myotoma appear as W`s and are separated from each other by a thin layer of connective tissue (myosept) as shown in figure 3. This configuration is important with respect to longitudinal separation of myotoma due to microbial or enzymatic decomposition (gaping), or thermal decomposition of binding tissue (flaking). Flaking is one of

the challenges which must be considered when performing studies on texture.

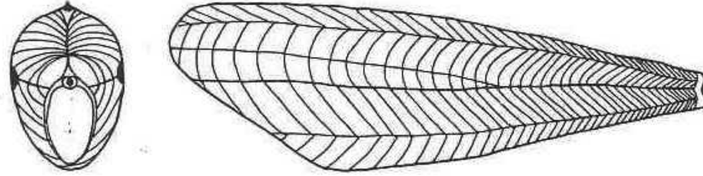

Figure 3: Structure of cross and longitudinal sections of fish fillet. Lines representing connective tissue, myocommata, separating one block of cells, myotoma, from another (Ofstad, 1995).

1.2.1. Quality attributes of farmed cod

There are some important differences between wild and farmed cod. Farmed cod is characterised by a high liver-somatic index (Gildberg, 2004) and a high glycogen content (Einen et al., 1999) which results in a low ultimate pH post-mortem (Rustad, 1992). A low pH easily leads to gaping (Haard, 1992), high liquid loss and reduced WHC (Ofstad et al., 1996a) and altered muscle texture (Love, 1979; Ang & Haard, 1985; Losnegard et al., 1986; Segars & Johnson, 1987; Einen et al., 1999; Kristoffersen et al., 2006). However, after cooking, the texture of farmed cod may have a rubbery and meaty texture compared to wild cod (Haard, 1992; Sveinsdottir et al., 2010). Because of the faster growth, farmed halibut has small myofibrillar units between the larger ones, which has not been observed in wild halibut (Olsson et al., 2003) which could be expected for cod as well. More small fibres are found in fast growing strains than in strains that grow slowly. By sensorial analyses it has been shown that increasing fibre diameter result in lower scores for firmness and chewiness (Hurling et al., 1996; Johnston et al., 2000).

There are also several visible differences between wild and farmed cod; the farmed cod has darker skin, smaller head and also look more “stuffed” due to the larger liver. The fillet of a farmed cod is whiter

than a fillet from wild cod and the fillet is also thicker except at the stomach which is thinner.
As for any processed food, the quality of cooked fish depends on raw material quality. There has been a long tradition in Scandinavian canning industry to use frozen fish, mostly *post-rigor* filleted. Wild fish has to some extent been frozen or gutted and filleted offshore *pre-rigor*, while the rest have had to be landed before processing, i.e. *post-rigor*. Due to lack of a stable raw material supply, industrial production has been based on frozen fish. This has put some limits to the possible end quality of the finished products. Today several farmed species are available, and there are already three decades of experience on large scale farming of salmon and trout. Closed life cycles have been developed for haddock, pollock and hake, but production of these species have been rather modest while other species, e.g. cod and halibut have been farmed at an industrial scale since 2006 and are expected to grow fast in the coming years (Rosenlund & Skretting, 2006). This has also made a more stable supply of *pre-rigor* fish possible for those packaging and heat processing fish.

The pre-processing must also be designed to avoid degradation of quality. Several parameters are of great importance to the fish quality, e.g. handling, microbial contamination and temperature during processing and storage.

There are several methods for determining fish freshness; Sensory, microbial, physical and biochemical methods (Huss, 1995; Olafsdottir et al., 1997).

Sensory analysis cover a wide range of spoilage criteria and is therefore often more feasible than instrumental methods. Among the sensory methods available the quality index method (QIM) is currently the most widespread method and is also adapted to cod (Bonilla et al., 2007). While QIM is conducted on raw fish, sensory analysis may also, in difference to most instrumental methods, be conducted fish prepared by a standardised cooking method. The effects of the cooking method are not to be underestimated, especially when comparing ready-to-eat products of fish.

Microbial contamination is commonly used as indicator for fish spoilage. Guidelines and standards have presented limits for acceptable total viable counts (TVC). However, the detection of specific spoilage organisms (SSO) like *Shewanella putrefaciens*, *Pseudomonas* ssp. and *Photobacterium phosphoreum* is considered more reliable than total viable counts (TVC) to accurately evaluate the freshness or spoilage level of fish products (Gram & Dalgaard, 2002). Rapid microbial methods have recently increased the feasibility of microbial studies for evaluation of fish freshness.

Several chemical methods have to be mentioned. Ethanol has been used for quality determination as it is a common metabolite of a variety of bacteria. It may also be used for measurement of canned fish to reveal degradation of the raw fish prior to canning.

Peroxide value (PV) for measuring oxidative rancidity is a commonly used method in laboratories to evaluate fish, however not so applicable fish muscle of low fat content.

Another biochemical indicator for freshness of cod is the content og trimethylamine (TMA). Trimethylamine oxide (TMAO) is found in high concentrations in several marine fish. It is reduced to TMA by enzymes or spoilage bacteria (Malle et al., 1986), resulting in the characteristic smell of iced fish (Pedrosa-Menabrito & Regenstein, 1990). In Norway, TMA measurement has been implemented as an indicator of freshness by the Directorate of Fisheries and there is a maximum permitted level of 5 mg TMA-N/100 g in lean fish species. Wild cod, which is known to have high levels of TMAO in the muscle tissue (Hebard et al., 1979; Treberg & Driedzic, 2002; Esaiassen et al., 2004), is one of the species covered by this legislation. While the TMAO content of wild cod is 70-80 mg TMAO-N/100 g muscle it is only 10-20 mg TMAO-N/100 g farmed cod muscle and it is therefore suggested that TMA is not suited as indictor for freshness of farmed cod (Herland et al., 2009).

Among the physical methods used for evaluation of fish freshness, determination of drip loss and WHC are rapid and widespread methods. WHC is discussed in detail later in thesis. An obvious limitation for freshness evaluation is the fact that WHC is influenced

other parameters, e.g. pH, which is also changing during storage, especially during *rigor mortis*. pH may, or may not change as a result of microbial activity.

Electrical properties of fish are changing during *post mortem* storage. However, the electrical properties depend on several other parameters as well as the fish freshness, e.g. physical damage, temperature (frozen or not) and bleeding procedure. Different systems are developed and have shown to give consistent results, e.g. the GR Torrymeter and the RT Freshmeter (VazPries et al., 1995). Dielectric properties have shown to be a measure which correlates to protein denaturation (Bircan & Barringer, 2002). Both the dielectric constant and loss factor increased at a temperature that appeared to match the DSC denaturation temperature for collagen in beef, chicken breast, chicken thigh, perch, cod, and salmon. When the sample was reheated the change did not reoccur, indicating that the dielectric properties were measuring an irreversible change. At lower frequencies the increase in the dielectric properties was larger. When collagen and actomyosin denature, the muscle shrinks, expelling water and minerals. This makes the water and ions more mobile. The dielectric constant and loss factor measure the mobility of water and ions; therefore they may be able to determine the temperature of protein denaturation. Dielectric might be of interest also for determining heat denaturation effects as well as denaturation due to spoilage of fresh fish.

Texture is a very important property of fish meat and several methods are available to determine fish quality. Variants of a compression test or a sharp blade test are the most common ones. Compression test are done either by a penetrating cylinder or a compression plate. Several publications exist on texture measurement of uncooked cod, but not on the cooked material. Because of the flaking of the material, compression tests usually is associated with scattering results. Separation of large flakes also occurs long before the general dcomposition which results in the fish falling into small pieces. The found publications do not make any distinction between the mechanisms of flake separation and cell separation. Test methods with one, five or ten blades which are forced through a fish sample have

been used in several studies, e.g. the Kramer shear-compression or Warner-Bratzler shear cell (Jonsson et al., 2001).

The texture of cooked cod is significantly influenced by the pH of the raw material as stated by Rustad (1992). Experience from high pressure processing may also be of interest to the topic, as a combination with thermal processing is often discussed. With a combination of cooking and pressure treatment, (Angsupanich & Ledward, 1999) showed that the hardness decreased to the same values as for cooking alone. They also concluded that heat treatment decreased chewiness, gumminess and hardness, but not the springiness and they suggested some reasons for this.

Presence and activity of the microbial load is closely related to freshness determination. In a recent work (Lin et al., 2006) it is demonstrated how NIR spectroscopy could be applied to detect and monitor microbiological spoilage in rainbow trout. In this work the microbial load was measured as total viable count. Despite that short-wavelength NIR (600 – 1100 nm) is considered not applicable to directly detect bacterial cells (Lin et al., 2006), the different spoilage bacteria produce characteristic compounds making the microbial prediction possible from the spectral data: H_2S-producing organisms, like *S. putrefaciens*, develop sulfur compounds contributing to off-flavour, growth of bacteria like *S. putrefaciens* and *P. phosphoreum* are accompanied by development of ammonia-like and 'fishy' off-flavors due to reduction of TMAO to TMA, while *Pseudomonas*, in particular *P. fragi*, often are responsible for quality changes and development of sweet, fruity off odours in chilled fish (Olafsdottir et al., 2006). Other reports show how spectroscopic techniques may also be used for pathogen identification (Al Holy et al., 2006; Al Qadiri et al., 2006).

1.2.2. Thermal properties of cod muscle

The thermal properties of cod are of great importance for modelling heat transfer but have only scarcely been reported in literature. Thermal conductivity and specific heat capacity are known for room temperature, but not for elevated temperatures. Conductivity data are often reported without information on content of fat and water or in

which direction of the cod muscle the data have been recorded. However, since the cod muscle has low fat content (0-0.3%), stabile water content and a fibre structure in a variable angle relative to the fillet, detailed information could not be utilised for engineering purposes anyway.

Thermal conductivity for foods are reported in several compilations (Rao & Rizvi, 1995). For most foods the thermal conductivity is in the range of 0.2 to 2.0 W/mK. For fish products a range from 0.2 to 0.4 W/mK is more typical.

For conductive products it is common to use thermal conductivity k (W/mK) for calculation, defined from the Fourier law as (in one direction, x)

$$k \equiv \frac{q''_x}{(\partial T / \partial x)} \tag{1}$$

where q''_x is the heat flux (W/m^2)

General data are available from the ASHRAE Handbook of Fundamentals and Rao & Rizvi (1995). K of fish can be modelled by

$$k = 0.0324 + 0.329 \cdot W \tag{3}$$

where W is water content in decimals. For cod, water content is ~80%, thus, the model gives a thermal conductivity of 0.30 (W/mK). Sweat (1995) suggested this best-fit equation for food in general based on 430 point found in literature:

$$k = 0.58 \cdot X_w + 0.155 \cdot X_p + 0.25 \cdot X_c + 0.16 \cdot X_f + 0.135 \cdot X_a \tag{4}$$

where parts pr. 1.0 of

X_w water

X_p protein

X_c carbohydrate

X_f fat

X_a ash

An alternative to k is to use the thermal diffusivity, defined as

$$\alpha = \frac{k}{\rho \cdot c_p} \quad (m^2/s) \text{ where} \qquad (2)$$

ρ is density kg/m^3

c_p is specific heat capacity J/kg K

Density of cod (ρ) and specific heat capacity is known from Waterman (2001) to be 1054 kg/m^3 and 3347 kJ/kg K respectively at 0°C.

Using these data with equation 2 gives a thermal diffusivity of $1.19 \cdot 10^{-7}$ m^2/s. This is close to the thermal diffusivity measured to $1.22 \cdot 10^{-7}$ by Riedel (1969). The literature data on thermal conductivity and thermal diffusivity in cod muscle are scarce and should be confirmed by experiments.

Even less information is available on surface heat transfer coefficient from cod to surrounding heat transfer media, e.g. water or steam-air mixtures. None of the references obtained describe the actual situation with vacuum packaged cod in a steam cabinet or an autoclave. Thus, general models may be used or (preferably) own experiments should be performed.

1.3. Microbial constraints for mild heat treatment of cod

The quality of fresh fish is rapidly reduced as a consequence of various microbial, biochemical and chemical breakdown processes and it is important to perform the thermal processing of the fish before these breakdown processes results in significant loss of quality. The initial quality loss is mainly due to the *post mortem* autolytic activity and chemical degradation process, but from the mid stages of product shelf life, the microbial quality changes are increasingly important (Huss, 1995). Several psychrotolerant gram-negative bacteria (e.g.

Pseudomonas spp. and *Shewanella* spp.) grow on fresh chilled fish but may be inhibited by vacuum packaging in favour of *P. phosphoreum* and lactic acid bacteria as reviewed by Gram and Dalgaard (2002). These microorganisms are also associated with farmed Atlantic cod (Huss, 1995; Hovda et al., 2007). However, none of these spoilage bacteria are heat resistant and could not survive a mild thermal processing.

The quality of thermally processed products is highly depending on the heat load which is determined by the requirements for inactivation of microorganisms. Legislations on inactivation of pathogenic microorganisms often include a safety margin which may lead to over-processing of some goods. In the USA the food and drug administration (FDA) early announced concerns for the safety of pasteurized foods (Rhodehamel, 1992) and it has been common to distribute pasteurized fish products frozen. In the current recommendations from the FDA's "Food Code" techniques like sous vide and cook chill should only be used if the fish product is kept frozen from packaging/thermal processing and until consumed (US Food and Drug Administration (FDA), 2005). Safety of hermetically packaged and thermally processed foods has been an issue since the invention of the technology, and detailed legislations are in use. National legislations are often very different from each other, but there are also several things they have in common. For commercially sterilised products the legislations have been more focused on the safety of the end product. Sterilised foods have been used worldwide for several decades and one of the requirements most countries have in common is that the least sterilising value F_0 should be 3,0 for a low acid canned food. This general requirement has also been applied to fish. Internationally recognised guidelines are published by the United Nations Codex Alimentarius Commission for canned foods (2001). In most legislation for hermetically packaged heat preserved foods, the following topics, in addition to more general issues like hygiene, are of major concern:

- Determination of a safe heating procedure, i.e. requirements to sterilisation- or pasteurisation values.
- How to achieve the required sterilisation/pasteurisation, i.e. heat penetration tests or other measuring techniques, and to

determine a scheduled heating process (e.g. sterilising time and temperature)

- How to reproduce a scheduled process, i.e. control of heat distribution and constant heat transfer conditions in the product
- Validation of procedures and equipment (at least calibration of thermometers) and record keeping
- End product control. For sterilised products this includes incubation and microbial sample testing.
- Integrity of packaging

European Guidelines for canning have been published by the Campden and Chorleywood Food Research Association (CCFRA) (May, 1997). Volunteer organisations, like the National Food Processors Association (NFPA) and the Institute for Thermal Processing Specialists (IFTPS) have made several publications which are widely used (IFTPS, 1992; IFTPS, 1995; IFTPS, 2002). All these guidelines describe how to perform heat penetration tests in canned products and heat distribution tests in autoclaves. These are issues also important to milder heat preservation techniques, and several elements can be transferred from the canning guidelines. The guidelines for heat penetration tests of canned foods could be successfully used for almost any heating regime for packaged foods.

During the development of any new heat treated product it is essential to assess the combined effects of the total system consisting of heat process, preservatives, packaging and storage conditions in order to ensure that the product is of good microbiological quality and does not present any food safety hazard.

In the environments for fish several pathogens are indigenous. Some are toxin producing bacteria, such as psychrothrophic non-proteolytic *Clostridium botulinum* type B,E and F, and psychrotolerant histamine producing bacteria (photobacteria). Other relevant microorganisms are *Listeria monocytogenes*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *Plesiomonas shigelloides* (Nilsson & Gram, 2002). Fish may easily be contaminated by bacteria from the environment during processes like gutting and filleting. Psychrotropic *C. botulinum* type E and *L. monocytogenes* are

examples of bacteria that may easily contaminate fish. Pathogenic toxin producing *Bacillus cereus* is not associated with raw fish materials, but may be a risk factor from the ingredients in mixed or minced fish products or in marinades (Feldhusen, 2000). *C. botulinum, Vibrio spp.* and *L. monocytogenes* constituted the highest proportion of outbreaks origin from seafood as reported internationally in the period 1988 to 2007 (Greig & Ravel, 2009). In summary, a thermal process should target a safe destruction of these pathogens if growth of these bacteria cannot be inhibited during the product shelf life, e.g. by refrigeration or preservatives.

Bacterial spores and their heat resistance have been focused on in several studies (Setlow & Johnson, 1997; Lindstrom et al., 2006; Peleg et al., 2008; Rajkovic et al., 2010; Silva & Gibbs, 2010). Spores formed by the genera *Bacillus, Clostridium, Desulfotomaculum* and *Sporolactobacillus* are hot topics in food microbiology and *Clostridium* spp. receives special attention in relation to thermal processing. Several guidelines and code of practice have been published with respect to safe production of ready to eat packaged foods with extended shelf life under refrigeration (ACMSF, 1992; Betts, 2009; ECFF, 1996; ACMSF, 2006). Most of these are targeted at preventing growth and toxin production by non-proteolytic *C. botulinum*. A general recommendation in the guidelines mentioned above is that the heat treatments or combination of processes utilized, should reduce the number of viable spores of non-proteolytic *C. botulinum* by a factor of 10^6 (6D). Accordingly, a minimum heat treatment of 90°C for 10 min or equivalent lethality in the slowest heating point of the product has been recommended by ACMSF (1992, 1995). This is based on D_{90} of 1.6 min and a z-value of 7.5ºC when the temperature in the product is below 90ºC and a z-value of 10ºC at higher temperatures.

L. monocytogenes is a Gram-positive bacterium, mobile by means of flagella and has been considered as a leading cause of death amongst the food borne bacterial pathogens (Paoli et al., 2005). Epidemiologic data indicate that foods involved in listeriosis outbreaks are those in which the organism has multiplied and in general have contained levels significantly higher than 100 cfu/g (Buchanan et al., 1997; ICMSF, 2002).

The Codex Alimentarius recommended as well that the maximum contamination level for *L. monocytogens* in food at consumption should be less than 100 CFU/g based on risk assessment for *L. monocytogenes* in RTE foods (Codex Alimentarius, 2002).

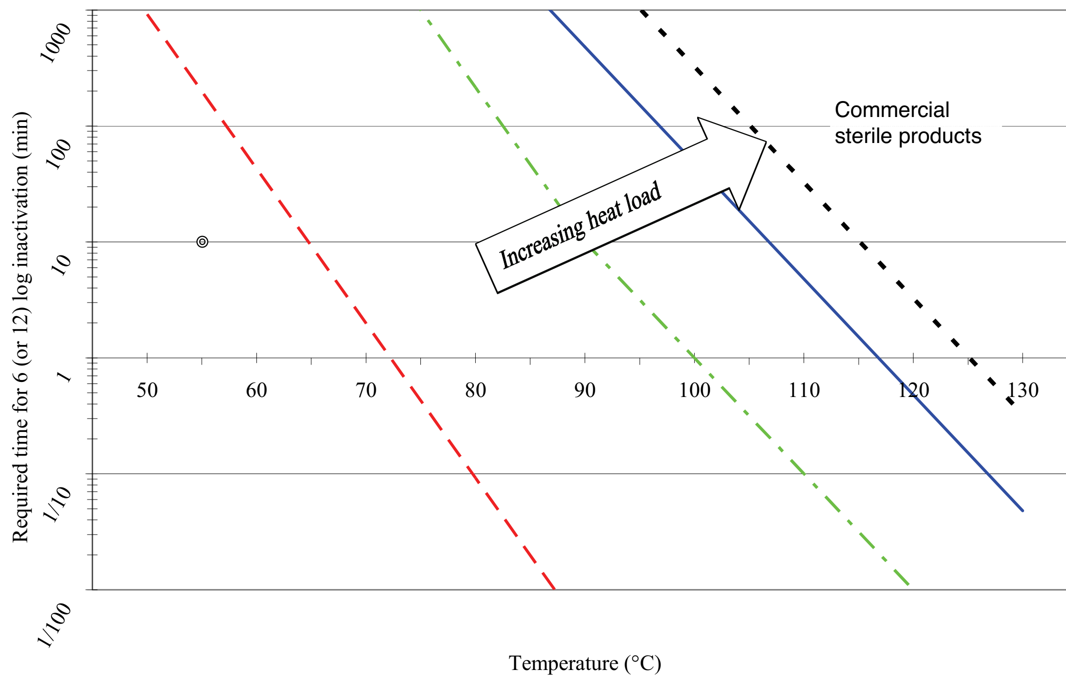

Figure 4. Required heat load for 6 log inactivation of some target organisms from left to right; *V. parahemolyticus* (double circle), *L. monocytogenes* (dashed line), psychrotropic non-proteolytic *C. botulinum* type E (dashed/dotted line), *B. cereus* (solid line) and *C. botulinum* type A (12 log inactivation, dotted line). The area on the right hand side of each line represents what is recognised as the "safe side" for that organism.

As mentioned above, the 6 D concept is also applicable for *L. monocytogenes*. Accordingly, a minimum heat treatment of 70°C for 2 min or equivalent lethality in the slowest heating point of the product has been recommended by ACMSF (1992, 1995). This is based on a $D_{70°C}$ of 0.33 min and a z-value of 7.5ºC. There is, however, a wide range of kinetic data reported for inactivation of *L. monocytogenes* depending on the strain and the model system used for determining

the heat resistance (Ben Embarek & Huss, 1993). For cod, Ben Embarek & Huss (1993), investigated the heat resistance of *L. monocytogenes* O62 and found a D_{70} of 0.03 min and a z-value of 5.7, while he found a D_{70} of 0.05 min and a z-value of 6.1 for *L. monocytogenes* O57. This indicates that a 6 log inactivation would require a pasteurisation value $P_{70°C}$ in the range of 0.18 to 0.30 min, but further studies of heat resistance of *L monocytogenes* in fish are needed to draw a conclusion.

The heat resistance of *V. parahaemolyticus* has been reported by several authors as reviewed by Drake et al. (2007) but kinetic data for thermal inactivation are scarce; only one detailed report on D-value stated a $D_{55°C}$ of 1.75 min (Johnston & Brown, 2002) and z-values are only found for other *Vibrio* spp. Based on this and the internationally recognised heat resistance for some of the target organisms mentioned, the required heat load for 6 log inactivation is shown in figure 4 as based on kinetic data discussed above.

The data in figure 4 are reported for log-linear inactivation kinetics, except for non-proteolytic *C. botulinum* type E which has a break point at 90°C on the inactivation line and *V. parahaemotolyticus* where only one point is shown. In recent years the commonly used first order inactivation models described above has been challenged by more sophisticated non-log-linear modelling (Peleg & Cole, 1998; Peleg, 2006). This might give opportunities for more accurate optimisation of thermal processes in the future if this can be adopted in legislations and standards, and more detailed knowledge is gained. Another aspect challenging the log-linear inactivation models is the detection of higher heat resistance of bacteria on the surface of foods compared to free-floating microorganisms (Lejeune, 2003). At the surface of the fish it is also expected to have a much higher microbial load than inside the fish meat which together with a higher heat resistance could make a problem. On the other hand, the heat load is much higher on the surface of the product compared to the core when using conventional heating systems. For rapid heating technologies this is not always the case. Heat resistance of *L. monocytogenes* on a Teflon surface during steam pasteurisation has also shown to follow other patterns than all previously suggested inactivation models (Valdramidis et al., 2007). However, for a conventional pasteurisation

in an autoclave or steam cabinet, the well established log-linear inactivation kinetics and calculation of pasteurisation values in the core of the vacuum packaged fish product is still the method that is in practice.
Even for a product intended for immediate consumption after cooking, e.g. fresh fish cooked and served in a restaurant or in a home it is recommended by the FDA-Food Code to heat the fish to minimum 63ºC for 15 s for food safety reasons (*Salmonella spp).* However, some chefs and cookbooks recommend using temperatures in the range 48 °C to 60 °C to achieve the desired quality. Temperatures below 55°C may even be insufficient for killing nematodes (Huss, 1994). Nematodes may be avoided in farmed fish e.g. in salmon (Lunestad, 2003), but have still been found in farmed cod (MacKenzie et al., 2009).

In conclusion, two levels of heat load can be used as threshold for refrigerated vacuum packaged fish preserved by heat:

1. A mild thermal process designed to inactivate *L. monocytogenes*: $P_{70}^{7.5} > 2$ min resulting in shelf life within the range of 10 days for storage below 4 °C or possibly longer if stored below 3 °C.
2. A thermal process designed to inactivate spores of non-proteolytic *C. botulinum*: $P_{90}^{7.5} > 10$ min resulting in shelf life within the range of 21 days for storage below 4 °C.

These safety levels should be respected as long as a milder heat process has not been proven to be safe. The only feasible alternative for extension of the shelf life for the products in question is frozen storage.

1.4 Technological aspects of mild thermal processing

The recent advances in minimal processing of fish and its implications on microbiology and safety have been reported and give an overview of both processes and methods for mild thermal processing (Rosnes et al., 2011).

1.4.1 Equipment and validation methods

For pasteurisation at temperature from about 90°C and up, a counter pressure may be desirable for flexible packaging materials and in some cases (e.g. easy peel top film) even necessary. This result in the need for an autoclave, but even at temperatures below 90°C, the autoclave may be the preferred solution because of the possibility of counter pressure and a temperature distribution that normally is much better than the alternatives. The pressure may also be of importance to the heat transfer and the safety of the product (Skipnes et al., 2002). A low pressure may result in a dead space between the food and the packaging and insulate the food. A sudden pressure change at start of cooling may result in ebullition and an unexpected fast temperature fall inside the product.

Alternative equipment for mild heat treatment is water immersion and steam cabinets. As for autoclaves, these solutions have their continuous variants with steam tunnels and water baths with conveyors. For a water bath with sufficient circulation (at least 50% exchange of water per min) and spreading system, a temperature distribution comparable to a modern autoclave should be possible. For cabinets, the performance depends on the mixture of air and steam in the cabinet end the fan system. Large temperature deviations must be expected in cabinets (Sheard & Rodger, 1995), and the variations in steam/air ratio may also result in uneven heat distribution also for seemingly acceptable temperature distribution. Measurements reported by (Nicolaï, 1994) revealed oven temperature differences up to 15°C between different locations for a pre-set temperature of 70°C

in a combi-steamer, using a hot air/steam mixture. There are usually also larger deviations from the set point temperature in cabinets than in autoclaves, but there are examples of improvements by advanced control strategies being used in spite of an inhomogeneous temperature distribution (Ryckaert et al., 1999; Verboven et al., 2000a; Verboven et al., 2000b). The large production of cabinets for kitchens has resulted in moderate prices, but for industrial fish processing, cabinets of high capacity and temperature uniformity within 1ºC is required.

Rapid heating methods are more suitable for continuous processing. Microwave heating still suffers from problems with uneven heating and limited penetration depth (a few millimetres) (Ryynänen, 2002).

Validation of both the scheduled process and heat processing equipments ability to deliver the scheduled process is crucial for the safety of the products. The most common way to do this is by temperature measurements inside the product (heat penetration test) and measurement of the temperature distribution in the equipment. The heat penetration curve obtained from the cold spot of the product is then used in eq. 6 to integrate the achieved lethality if the heat inactivation kinetics of the target organism is known. However, in several cases it is difficult to measure temperature (e.g. products agitated during heating) or even impossible (e.g. continuous systems) (Tucker et al., 2002) and in some cases the cold spot location is not fixed (e.g. microwave heating). Alternative methods for thermal process validation are therefore needed.

The use of time temperature integrators has gained popularity over the last decade. One practical result of this is the use of small beads to be placed within the food and analysed after thermal processing, giving either an indication of the maximum temperature reached or even the achieved lethality as given by the P (or F) value. Such time temperature integrators can also be used for studying factors of quality. For an overview on these very interesting and promising techniques, please refer to Hendrickx et al. (1995), Van Loey et al. (2004) and Tucker (2008).

Some time temperature integrators may be based on chemical or biological indicators intrinsic in the food product. If the biological activity or the concentration of the chemical marked is occurring in the food in a known concentration and its degradation kinetics is known, the heat load on the product may be determined after the heating process without any preparations. The application is the same as for other time temperature integrators, but may in addition be useful in cases of outbreaks of food illness or for regulatory purposes.

For pasteurised products the thermal processing must also take into account the desired shelf life of the product. The shelf life depends on:

- Time for surviving microorganisms to germinate and reach undesired levels or produce toxin.
- Time for enzyme activity or other chemical reactions to degrade the product to unacceptable level. For mild heat treatment the processing might even accelerate the activity of some enzymes.
- Physical factors, as discolouring by light etc.

A shelf life study must be done to validate the shelf life of the products. This study should include an evaluation of sensory, chemical and microbiological parameters of a product stored at or above expected realistic temperatures for a period longer than the expected shelf life. Microbial modelling may be used in addition to a shelf life study and is often a good tool in the early stages of product development. Software for microbial modelling is available today both as commercial and free software and should be carefully evaluated as there always will be an uncertainty related to such models. Therefore, Dalgaard, Buch & Silberg (2002) suggested a bias factor for seafood spoilage microorganism that should be between 0.75 and 1.25 for a microbial spoilage model to be successfully validated but no generally accepted criteria for successful validation of predictive models are available, at present. However, modelling of specific microorganismes and growth medium, e.g. *L. monocytogenes* in cold smoked salmon, has successfully been done (Dalgaard et al., 2002).

1.4.2 Quality optimisation by calculations and other methods

Ever since the invention of thermal processing the focus has been on how to minimize the thermal damage on the product (with the combination of microbial inactivation). An obvious approach to optimise the quality is sensory evaluation of a range of time/temperature combinations that result in the desired safety (F value). This is time consuming and expensive, but also gives the opportunity to optimise directly in terms of consumers of the product and as long as the only concern is to find the optimal time and temperature combination, this might be a feasible approach. The quality changes during storage are not to be forgotten when optimizing the thermal processing of a product.

Sophisticated methods for optimisation, including TTI's and computational fluid dynamics, have been introduced, as summarised in (Richardson, 2004). One of the easiest ways of performing optimisation is to take advantage of the cook value (C-value) as described above calculate the C-value for several time/temperature combinations that result in the same F value. The time temperature combination resulting in the lowest C-value correlates to the best quality retention (Tucker, 2003; Richardson, 2004). Quality changes are often to complex to be described in terms of a z value, but there are exceptions. For instance, the denaturation enthalpy of fish proteins during heating has been studied by differential scanning calorimetry.

Another limitation to the end quality of the product is given by the package geometry. The distance to the cold spot [1] of the product is crucial for the time necessary to achieve the desired sterilisation/pasteurisation through the whole product. The thermal conductivity of fish products depends on several parameters, and most important is the water content. The order of magnitude of thermal conductivity of fresh fish is 0.5 W/m K. For a solid product heat is transferred by conduction. At the boundaries of the product the

[1] The cold spot of a product is the place where the temperature is lowest during heating. This is not necessarily the geometric center of the product and has to be determined for each product.

temperature will be close to the water temperature during the heat treatment, resulting in a much higher heat load than at the cold spot.

Producers desiring a reproducible quality should keep in mind that continuous changes in the fish as raw material cannot be avoided, and therefore optimisation should be regarded as a dynamic process to be updated on a regular basis or even for each product batch. Thus, the optimisation tools need to be easy to use and give rapid results or even on-line process control.

1.4.3 Quality and effects of rapid heating

Thermal processing of foods (e.g. packaged in cylindrical cans) with conventional methods results in a much higher heat load on the surface compared to the centre of the product and a number of methods may be used to reduce the difference between surface and cold spot. It is often desired to achieve a similar temperature curve in the centre as on the surface as this would reduce the total heat load on the volume average of the product. In such cases the processing time is adjusted to achieve the required lethality of a target microorganism. This effect has been demonstrated by using cans of different diameters and it has been shown that the optimum processing temperature is decreasing when the can diameter is increasing (Ohlsson, 1980).

Rapid heating can be achieved in many ways than reducing the size of the food product. For liquid or semi-liquid products rotation of the package during conventional heating is very effective (Eisner, 1988). Conventional heating can also be speeded up by vibrations or by employing ultrasound to the product. However, for some fish products shaking, rotating etc. will make the fish fall apart and the use is therefore limited.

Rapid volumetric heating has been suggested as a method for reducing the heat load on the volume average of the product. Ohmic heating is a method that can generate temperature though an electrically homogen product, but the need for electrodes in contact with the food limits the method to aseptic packaging.

Heating with electric fields is more convenient for packaged products as long as appropriate packaging is used, like plastics. Use of metal containers is limited. Microwaves and radio frequency is more commonly used for thawing or tempering of foods than for pasteurisation or sterilisation in the food industry. Microwaves penetrate only a few millimetres into fish products and, thus, results in large temperature gradients through the cross section of the product. Radio frequency is penetrating several decimetres into the product, due to the longer wavelength, but may also result in uneven heating, especially if ions or minerals (like salt) are present in the product (McKenna et al., 2006). For Radio frequency heating, water immersion of the product can be used to stabilise the temperature at the product boundaries and may result in a more widespread industrial use in the future (Skipnes et al., 2003). Reduced cook loss and better controlled texture attributes are proposed in the ongoing research (Skipnes & Pfeiffer, 2005; Lyng et al., 2007). There might, however, be some limitations to the effect of rapid heating. Protein denaturation will typically be a process of low z-value, i.e. a small change in temperature will result in a great change in the time required for denaturation of proteins. Thus, changes in cook loss and water holding capacity will be highly influenced by process temperature while process time becomes less important. Most of the target microorganisms will show a higher z-value and therefore a low temperature at and relatively long process time will be favourable. For a process where this is the case there is little to gain on rapid heating. An example of such a situation is shown by Kong et al. (2007b).

1.5. Changes during heat processing

Aitken and Conell (1979) reviewed the effects of heating on fish and reported that the cooking losses varied greatly with the fish species, the method of heating and the heating regime (i.e. sterilization, pasteurization etc.). There are, however, few publications discussing the effects of heat on cod, the differences between thermally processed farmed and wild cod and the effects of filleting and heat processing pre- and *post-rigor*.

To the consumer the most obvious quality parameters are flaking (whether the fish is falling apart on the plate or not), tenderness and juiciness. From this perspective, the two main issues are the heat denaturation of proteins and the water holding capacity (WHC).

Heating converts the translucent, jelly-like cellular mass into an opaque, friable, slightly firm and springy form. The muscle is shrinking during heating, resulting in release of liquid. The proteins in this liquid may coagulate on the surface of the solid fish as a curd. The connective tissue holding the cells together is easily damaged and thus, cooked fish easily falls apart and becomes palatable on mild heating (Parry, 1970). *Post-rigor* processed fish does not fall apart as easily as pre-*rigor* processed, but it is more likely to brake across the myotoma. Even at temperatures as low as 37°C, the tensile strength is reduced to zero for codfish after 30 minutes (Aitken & Connell, 1979), and a visible softening of the connective tissue occurs after 15 min at 35°C. Below this no temperature effects were found by Aitken & Connell. However, Howgate & Ahmed (1972) showed that thermal effects on proteins were the most important parameter for texture in their study of drying of cod and hilsa at 30°C. Mullet fish was studied during cooking for 20 min in boiling water and showed to result in an increase of denaturated proteins from 12.82% to 27.15% (Aman, 1983).

Approximately 95% of the water in muscle is mechanically immobilised water, often referred to as “free water”. This water is free to migrate throughout the muscle structure and is of interest when measuring WHC in muscle. The fat content of cod muscle is in the order of magnitude of 0.3%. It has been reported that the average total liquid loss for non-fatty fish species is 18.6% (Aitken & Connell, 1979). Thus, the liquid loss must consist mainly of water and dissolved proteins. The nuance between water and liquid holding capacity is therefore less important for non-fatty fish species and WHC is in this text used to cover all liquid released from the fish even if it contains dissolved proteins and small amount of fat.

The major part of a fish muscle is segmented in myotoma formed like a disc that constitutes a separate muscle. These discs are mainly fibres that are single cells with a membrane named sarcolemma (Bremner &

Hallett, 1985). The fibres are composed of the myofibrils which contain the contractile sarcomere of actin and myosin in a cross-linked structure. Approximately 0.1% of the total tissue water content is chemically bound water, 5-15% is immobilized in the myofibrils, while the rest is extra-myofibrillar water which is the most critical for the WHC (Olsson, 2003). The extra-myofibrillar water is located between the myofibrils and between the myofibrils and the cell membrane (sarcolemma), between muscle cells and between muscle bundles (groups of muscle cells) (Huff-Lonergan & Lonergan, 2005). The ratio between the immobilised myofibrillar water and the extra-myofibrillar water is important as the latter is easily lost.

It is generally accepted that the forces that immobilise "free water" within the muscle are generated by surface tension (Hamm, 1986). More specifically, the water is trapped within the muscle by capillary action generated by small pores or "capillaries" (Trout, 1988). The pores producing the capillary forces are located between myosin and actin, and measures up to approximately 10 nm under normal conditions (Hermansson, 1983). Changes in the volume of the myofibrils will induce changes in water held by the muscle. In raw meat, absorption of water occurs by the entry of water into the myofibrils. Conversely, loss of water occurs by expulsion of water from the myofibrils as they shrink when filaments approach. WHC depends on heat-induced structural changes, sarcomere length, pH, ionic strength, osmotic pressure and state of *rigor mortis* (Ofstad et al., 1996a). Changes in WHC of farmed cod is only broadly related to bacterial growth (Olsson et al., 2007).

WHC of cod during heating has been studied by (Ofstad et al., 1993), who showed that the main structural changes occur in the connective tissue at low temperatures (<40°C) for both cod and salmon and they concluded that water loss at these temperatures is mainly due to denaturation and melting of collagen. These authors used a heating rate of 1ºC/min to the required temperature and a 10 min holding time. The solid material was placed on a net in a sample holder and centrifuged at 210 x g for 15 min. It was found that maximum water loss was attained when the muscle cell had shrunken due to denaturation of myosin and the extra-cellular spaces were widened.

The centrifugation loss decreased as a function of temperature when extra-cellular, granulated material became visible. Ofstad et al. (1993) found the centrifugation loss to be at maximum between 40°C and 60°C while it was lower at temperatures above 60ºC.

The properties of comminuted fish are affected both by the structure of the biological tissue and the new structures formed during processing (Ofstad et al., 1996b). However, as the changes in microstructure showed a close relation to WHC of the tissue during heating for coarsely chopped muscle, it was claimed that this would also be the case for comminuted fish. As expected, it was also found that increasing temperature from 30°C to 60°C increased the liquid loss. Ofstad *et al.* (1993) showed that liquid loss at 60°C was much higher when the muscle was comminuted before heating.

When heating salmon at 121.1ºC the cook loss increased rapidly to 14% after 5 min heating and then increased more slowly to 20% after two hours (Kong et al., 2007b).

For a solid portion of fish, e.g. a fillet cut of 140 g, during a conventional heating process in a steam cabinet, a temperature gradient from the surface to the core of the fish will exist. This has been studied for transport of moisture in tuna muscle. Bell et al. (2001) found that moisture transport out of the tuna muscle primarily resulted from the denaturation of muscle proteins and the resulting pressure gradient. The changes in mass transfer properties in the near-surface region reduced the mass loss rates but enforced the pressure gradient. The ratio of surface area to total volume is not expected to be of importance for a vacuum packaged fish portion.

The most labile muscle proteins of fish are collagen and α-actin. Collagen denaturation starts at about 30ºC and α-actin becomes insoluble at 50ºC. Any temperature sufficient to cook cod is sufficient to disrupt collagen so that it is not a factor in the toughness of the cooked material. Myosin becomes insoluble at about 55ºC and actin at 70-80ºC. Tropomyosin and troponin are the most heat resistant and become insoluble at about 80ºC (Hultin, 1996).

Muscle proteins are generally classified as sarcoplasmic (water soluble), myofibrillar (salt soluble) or stromal (insoluble) (Skaara & Regenstein, 1990), of which the myofibrillar constitute the largest fraction of the total protein in cod (76%), and the stromal (extracellular), fraction, which mainly consists of collagen, the smallest (3%) (Suzuki, 1981). Cod muscle contains a large number of proteins and 50% of the 446 unique proteins identified by Gebriel et al. (2010) were assigned general binding functions.

The sarcoplasmic proteins as such, do not contribute much to texture changes because they have a very low capacity for immobilization of water in their structure (Dunajski, 1979). The enzymes present in the sarcoplasmic fraction, however, may influence gelation of intact muscle.

Myosin and actin are the myofibrillar proteins directly involved in contraction-relaxation. Due to the low collagen content of fish, their role in gelation and texture is even more important than in meat (Brown, 1987). Much work has been done on thermal gelation of myofibrillar proteins, especially related to surimi (Lanier, 1986; Gill et al., 1992; Arai, 2002). The special mixture with salt, however, and the fact that the studies of surimi focus on low temperature gelation or "setting" caused by transglutaminase activity (Tsukamasa & Shimizu, 1990; Tsukamasa & Shimizu, 1991), reduces their relevance for regular thermal processing of intact muscle.

Effects of proteolytic enzyme activity in fish products have been reviewed by Haard (1994) and Skåra and Olsen (2000). The problems related to residual enzyme activity in fish products after thermal processing, are mainly quality changes in the texture. Deng (1981) studied textural changes in mullet during heating and found different profiles in shear force when changing the heating process. He suggested that texture of cooked fish is tougher and is due to physical protein denaturation only, while a slower and/or stepwise heating process gave a tender/softer texture which he explained by alkaline protease activity. Most enzymes are inactivated at temperatures above 50°C (Svensson, 1977), while some enzymes are known to be heat stable (with remaining and stable activity over a certain range of time and temperature), and therefore have the potential to change the

product quality. In fact some heat stable alkaline proteases are more or less inactive at physiological temperatures and activated at high temperatures only (Dahlmann et al., 1985; Toyahara et al., 1987).

Cod meat of good quality has a mild taste which is not much altered by mild heat treatment. However a phenomenon well known from mammal meats, Warmed over flavour (WOF), has also been reported for fish. WOF sometimes develops 1-2 days after thermal processing and is characterised as the taste of carton or paint. WOF is caused by oxidation of lipids and often associated with mammal meats but also in relation to fish (Pearson et al., 1977). Thermal processing releases iron and other ions from the fish meat and increases the rate of the oxidation in the temperature range 60-70°C. Above 80°C, and especially around 100-110°C Maillard reactions will slow down the reaction as reduction of sugar and proteins have an anti oxidative effect (Yamauchi, 1972). Addition of antioxidant to the fish feed (e.g E-vitamin, alfa-tokoferol) will reduce the rate of oxidation. The development of WOF is commonly measured by the content of thiobibuturic acid (TBA). Incidences of WOF in cod muscle have rarely been reported, possibly due to its relative low fat content or regarded as rancidity developed during storage. However, formation of TBA during cooking of comminuted cod has been found and may be controlled by addition of chitosan (Shahidi et al., 2002).

1.5.1. Methods for analysing cook loss and WHC.

Kinetic data on WHC of cooked material was not found in the literature and a method for such analysis was not found, while methods for analysing raw material were numerous. WHC can be defined as the amount of water left after centrifugation relative to either the water present before centrifugation (WHC_w) or to the dry weight (WHC_d) (Andersen & Jørgensen, 2004). When referred to as liquid holding capacity the amount of expelled fat is also taken into account. For a non-fatty fish like cod, the loss of fats is negligible and WHC and LHC can be regarded to be equal.

In principle there are three methods commonly used for measuring WHC, which is:

- Nuclear magnetic resonance (NMR)
- Centrifugation
- Drip loss

Bertram et al. (2001) demonstrated significant good correlation between these methods by studying pork meat at different pH.

Drip loss is determined by keeping the sample on a net for a time ranging from 12 hours and up to several days. Honikel (1998) described a method where the sample is hanging in a net inside a plastic bag for 72 and determining the drip loss by weighing the sample before and after hanging. Because of the cook loss expelled during thermal processing further drip loss will be very limited and can not be used for characterising WHC.

NMR has been used by Jepsen et al. (1999) to determine WHC. They showed that the method also has the ability to characterise mobility of the water pools in three fish species including cod. Compared to the centrifugation method NMR is extremely expensive and time consuming.

Two varieties over the centrifugation method used for fish has been found, one based on (Eide et al., 1982) and the other on (Hermansson, 1986). Both are based on placing the sample in a tube with a net in the bottom and centrifuging the sample before a second weighing. Except from differences in pore size of the net, centrifugation speed, sample size and centrifugation time they are in principle the same.

The methods developed for characterizing raw material might also be used for cooked food by taking the cook loss into account in the calculation. However, this would require transfer of the sample from the apparatus for heating to the WHC analysing equipment. This is impractical and there is a risk of losing parts of the sample in this operation as the fish tends to stick to several surfaces. Preferably, the heating and cooling should be done in the pipes used for analysing WHC. This has been tried but was restricted to a heating rate of 1 °C/min (Ofstad et al., 1993). To obtain kinetic data, strict temperature

control is required and effects of time should be studied under isothermal conditions, i.e. heating and cooling should be done in much less time than the protein denaturation reactions. Thus previously available methods were not suitable for investigating the kinetics of heating induced changes in WHC.

1.6. Nutritional aspects of thermal processing

Thermal processing may affect the nutritional quality of foods both positively and negatively. Some nutrients, like most water soluble vitamins, are heat sensitive and might be reduced in amount or completely lost if the heat load is high enough. Further may the cook loss also contain both water soluble and lipid soluble vitamins. This cook loss may also contain water soluble proteins and should be investigated. On the other hand, heat treatment may increase the digestibility of the nutrients remaining in the cod muscle.

The major group of nutrients from the cod muscle is protein. Thermal processing below 100°C will not alter the composition of amino acids, but the functional properties might be changed (Cheftel et al., 1985). Further studies on this could be considered, as the literature specific to heat denaturation of cod or fish proteins is scarce.

In recent years there has been growing interest in biologically active compounds in foods, which are not regarded as essential nutrients, but can be beneficial under certain circumstances. One of those is taurine (2-aminoethanesulphonic acid) which is found in high concentrations in fish muscle (Lyndon et al., 1993). Both taurine and creatine are examples of beneficial compounds available in cod muscle. Taurine is an exclusively free amino acid (FAA) and may be regarded as conditionally essential (Niittynen et al., 1999). Boiling for 10 min reduced the taurine content of cod loins with 15% while heating to core temperature of 63°C for 15 s reduced the taurine content by 10% (Larsen et al., 2007).

Thermal processing in the range up to 100 °C is not expected to chemically change other nutritional components of the cod muscle, neither for minerals nor fatty acids.

In conclusion, there is not enough information available for optimising the thermal processing of convenience cod products with respect to nutritional outcome.

2. Main results and discussion

The raw material used for the following studies was farmed Atlantic cod (*Gadus morhua*). More specific, loins were cut from fillets of the fish after deep skinning and further processed before analysis. Except from the studies presented in paper I, the fish was of the same origin. This means that the fish used for the studies presented in paper II-V came from the same breeding station and were subjected to comparable feeding and life conditions. Most of the work was done on coarsely ground raw material mixed from a batch of 94 individual cod, except for part of the raw material presented in paper IV (which partly presented a seasonal study) and a part of the raw material presented in paper V (which partly presented data for individual fish).

The work presented here was part of a project covering other studies on fish of the same origin. This work includes several studies. Effects of brining on WHC where studied by Johnsen et al. (2009) on the very same coarsely ground material as mentioned above. The bacterial flora was characterised for cod from the same stain net as the fish described in paper II-V (Hovda et al., 2007) and microbial quality of cod from the same stain net was used for evaluation of microbial quality of desalted cod in modified atmosphere packaging (Rotabakk et al., 2009). Further, the raw material quality for fish from the same stain net was subjected to several studies with sampling partly coordinated with the seasonal study presented in paper IV (Herland et al., 2007; Herland et al., 2009; Herland, 2009; Herland et al., 2010). These references give the presented work a broader perspective as well as detailed background information.

Heat treatment of all samples was done in water baths. All of the papers I, III, IV and V present results from fish heated using a similar methodology which benefits is presented in paper I and in chapter 2.1.1. Paper II presents results from heating of extracts in glass tubes immersed in a water bath. Strict temperature control, homogenous temperature distribution and close to isothermal temperature conditions were exercised in all cases.

Please refer to figure 1 for an overview of the organisation of this chapter.

2.1. Food quality

As concluded from the previous chapter, texture and juiciness are the most obvious quality parameters to the consumers of cod loins and is therefore discussed in the following chapter.

One of the main objectives was to measure the water loss associated parameters during thermal processing of cod in terms of water content, cook loss and WHC. To the food industry this parameter is of importance for control of drip loss from the cooked food during distribution and in relation to juiciness of the fish. A method and associated equipment designed for combining rapid and homogenous heating with direct measurement of cook loss and WHC was developed and patented (Skipnes & Vilhelmsen, 2006). Further, the mentioned method and associated equipment was utilised when evaluating heat induced changes in colour and texture.

2.1.1. A method for characterising cook loss and WHC

The method was based on measurement of the liquid exudates from a fish sample during thermal processing and subsequent centrifugation. A new sample cup (figure 5) intended for combined heat processing and measurement of water holding capacity (WHC) and texture of fish material was developed. The analysis of WHC was based on the method described by Børresen (1980), but the centrifugation speed and time were modified and the new sample cup was used. The ability to conduct reproducible experiments using this sample cup has been demonstrated (I). Various centrifugation speeds used on previously developed sample cups were tested and it was decided to use a centrifugal speed of 528 x g. The calculation method was also modified for cooked fish material by including the cook loss in the calculations.

The method proved to give a relatively low standard deviation (<3.6%) when using a homogenous fish mince compared to the findings with earlier methods (<10%). For samples of whole tissue, the scatter in the results was too large to discriminate among the different heat loads on the samples, probably due to the variation between individual samples. Using the sample holder developed, the measurement of cook loss seemed feasible. It was demonstrated that cook loss is increasing with increasing temperature when farmed cod was isothermally heated for 10 min at different temperatures in the range 40ºC to 90ºC. In order to establish kinetic data for WHC (as a function of time and temperature), it seemed required to use a homogenous sample of ground meat.

After gravimetrical determination of cook loss, the cups were centrifuged and the water loss was measured in order to calculate the water holding capacity. The water content was determined from additional samples placed in a drying chamber at 105ºC for 16 hours (these samples were not centrifuged). The same method has also been used to study different variables, e.g. different raw material (I) and different brining, seasonal variations and time from slaughter (Johnsen et al., 2009).

Figure 5. Sample cups with filters and lids for top and bottom.

Three different centrifugation speeds used in earlier methods were included; 173* g, 326* g and 528* g for 15 minutes each. Weight loss was then registered. The standard deviation of the proposed method was tested with a homogenously minced fish. In this test, 8 samples were heated at 90ºC for 10 minutes, cooled and centrifuged together with 8 raw samples at 528* g for 15 minutes.

Liquid loss was measured for all the samples as the amount of liquid that passes through a filter in the bottom of the sample cup. The water holding capacity could then be determined by the weight of the liquid released. Figure 6 shows the composition of the samples divided into water and dry material. The initial weight of a raw sample m_0 is equal to the sum of the initial water content of raw material, V_0, and dry material, D_0. The weight of the exudates (the liquid separated from the sample during centrifugation) was named ΔV_0 for raw samples.

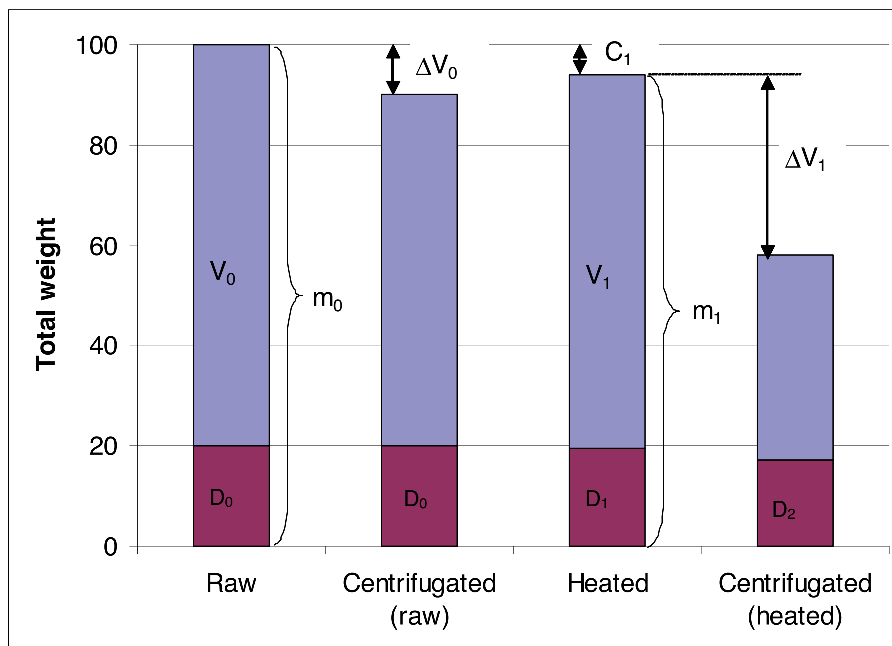

Figure 6. Weight of sample before (m_0) and after heating (m_1) split in dry material (D) and water content (V). Cook loss is shown as C_1 and centrifugation losses as ΔV_0 and ΔV_1 for raw and cooked material, respectively (adopted from I).

WHC of raw samples were calculated as percentage remaining water of initial water in sample;

$$WHC = \frac{W_0 - \Delta W}{W_0} \cdot 100\% \quad (5)$$

where,

$$W_0 = \frac{V_0}{V_0 + D_0} \cdot 100 \quad \text{and} \quad \Delta W = \frac{\Delta V_0}{V_0 + D_0} \cdot 100 \quad (6)$$

When the samples were cooked, liquid was expelled from the fish as cook loss, C_1. This cook loss consists of water, dissolved proteins, ash, salt and fat. The remaining dry material in the sample, D_1, will be in the range 0 to 6 % smaller than D_0. During centrifugation additional dry material is removed from the sample together with the centrifugation loss and the remaining dry material, D_2, will be significantly lower than D_0.

For cooked material a WHC_1 can be defined

$$WHC_1 = \frac{W_1 - \Delta W_1}{W_1} \cdot 100\% \quad (7)$$

where

$$W_1 = \frac{V_1}{V_1 + D_1} \cdot 100 \quad \text{and} \quad \Delta W_1 = \frac{\Delta V_1}{V_1 + D_1} \cdot 100 \quad (8 \,\&\, 9)$$

Thus,

$$WHC_1 = \frac{V_1 - \Delta V_1}{V_1} \cdot 100\% \quad (10)$$

By using this calculation method the WHC will describe the ability of the cooked material to withhold the water during centrifugation. Dry matter lost during the centrifugation step is included in the calculation. This is common practice as in practical terms it represents a real loss. However, the dry matter content may be determined either directly from the exudates or via the dry matter content of the centrifuged fish material and subtracted from the rest of the centrifugation loss. As the water expelled during cooking (before centrifugation) is not taken into account, the method will not describe neither the water loss nor the

protein denaturation very well. The method was improved by calculating the WHC on the basis of the water content of the raw material and including the cook loss in a total WHC (WHC_{TOT}) which is calculated by;

$$WHC_{TOT} = \frac{W_0 - \Delta W_{TOT}}{W_0} \cdot 100\% \qquad (11)$$

where,

$$\Delta W_{TOT} = \frac{\Delta V_1 + C_1}{V_0 + D_0} \cdot 100 \qquad (12)$$

which leads to a new definition of WHC

$$WHC_{TOT} = \frac{V_0 - (\Delta V_1 - C_1)}{V_0} \cdot 100 \qquad (13)$$

This calculation method describes the total change in water holding capacity from raw material to cooked product. As in the traditional way of calculating WHC, the dry matter content of the exudates is included in the calculation.

Freezing, followed by thawing, will result in rapid increase in cook loss and higher loss in WHC at lower temperatures than for fresh fish (I).

It is feasible to use the new method and associated equipment (sample cups) for rapid and uniform heating of samples and after heating and cooling gravimetrically determine the cook loss with a standard deviation within 30% of the total cook loss for minced samples. Both intact and coarsely minced muscle of farmed cod was used to investigate the abilities and limitations of the method.

2.1.2 *In situ* observations of quality changes

Water content did not decrease significantly when heating the ground fish muscle, even if a cook loss was observed (V). Thus, the content of dissolved proteins in the cook loss must be comparable to the level of proteins in the fish muscle. The total amount of water soluble proteins has been measured to 2.43% of the wet weight of farmed Atlantic cod muscle with a water content of 82.8 (Hultmann & Rustad, 2007). Assuming that half of these water soluble proteins may be dissolved in a cook loss of 10% of the loin weight; the amount of protein could amount to more than 10% of the exudates. This has been confirmed by observations of high protein content (ranging from approximately 4% to 14%) in drip loss from raw fillets of farmed Atlantic cod (Kristoffersen et al., 2007). Savage et al. (1990) revealed an average of 11% protein in drip loss from pork. This leads to the assumption that the protein content of the cook loss depend on the heat load and especially denaturation of sarcoplasmic proteins. Further studies on protein content in exudates from cod should be performed.

It is not known how much the loss of these water soluble proteins and the loss of water holding capacity affect the perception of juiciness. However, it has been shown by sensorial analysis that juiciness is increasingly lost with increasing heat load (McKenna et al., 2006), i.e. both juiciness and WHC is related to heat load but no documentation of correlation between juiciness and WHC has been found even if such a correlation seems likely.

In the following both cook loss and changes in water holding capacity is presented.

2.1.2.1. Cook loss

The cook loss was increased with increasing temperature, which is also the trend seen in other studies (Ofstad et al., 1993; Kong et al., 2007a). Even if these studies cannot be directly compared to the present study, they all confirm the trend of increasing cook loss with increasing heat load, i.e. the product of time and temperature. Even at temperatures as low as 20°C, water was lost from the sample after less than 10 minutes, and a cook loss appear immediately when heating to 90°C. Al combinations of time and temperature above 30 min at 90°C or higher resulted in more than 10% cook loss, while any combination below 80°C for less than 30 min restricted the cook loss to 5.6 %.

The primary mechanism leading to cook loss is thermal denaturation of muscle proteins (Bell et al., 2001) and heating of cod muscle has been observed to cause denaturation of myosin and shrinkage of myofibrils which leads to expulsion of water (Ofstad et al., 1993). However, the cook loss continued to increase when the samples were heated to higher temperatures than required for the proteins to denaturated and the water in the sample could be expected to be available for expulsion by a weak mechanical force. Such a mechanical force could be due to gradients of temperature, pressure and water concentration and will obviously lead to expulsion of water from any porous media that is heated, provided it contains free water.

Fish, like most foods, have small pores or capillary pores that may contain water. Most of the water is located within the myofibrils, in the narrow channels between thick and thin filaments (Offer & Trinick, 1983; Bertola et al., 1994). There are primarily three transport mechanisms for fluids in food; molecular diffusion (for gases), capillary diffusion (for liquids) and convection (pressure driven or Darcy flow). A mechanistic model of this fluid transport has been described by Ni, Datta and Torrance (1999) and have been presented in the equations for total flux of vapour $\vec{n}_v$ and liquid $\vec{n}_w$ (kg/m^2 s) as shown below (14,15). In addition an equation was developed for total flux of air, $\vec{n}_a$ which can be neglected in our case since the amount of

air in the vacuum pouch will be insignificant compared to the amount of water in the fish.

$$\vec{n}_v = -\rho_v \frac{k_g}{\mu_g} \nabla P - \frac{C_g^2}{\rho_g} M_a M_v D_{eff} \nabla\left(\frac{\rho_v}{P}\right) \qquad (14)$$

and,

$$\vec{n}_w = -\rho_w \frac{k_w}{\mu_w} \nabla P - D_w \rho_w \phi \nabla S_w - D_T \nabla T \qquad (15)$$

where C_g is molar gas concentration (mol/m^3), ϕ is relative humidity (% of saturated moist air), D_{eff} is effective capillary diffusivity (m^2/s), D_T is capillary diffusivity due to temperature gradient (m^2/s), M_a is moisture content (kg of water/m^3) with respect to air, M_v is moisture content (kg of water/m^3) with respect to vapour, P is pressure (Pa), S_w is saturation of water, T is temperature (K), k_w and k_g is permeability (m^2) of water and gas, respectively, μ_g is gas viscosity (kg/m s) and ρ_a, ρ_g, ρ_v, ρ_w is density (kg/m^3) of air, gas, vapour and water respectively.

Since air can be regarded as absent in the vacuum pouch, equation 14 may be simplified by neglecting the flux of vapour due to binary diffusion in air. Thus, the vapour flux may be calculated by equation 16.

$$\vec{n}_v = -\rho_v \frac{k_g}{\mu_g} \nabla P \qquad (16)$$

The water saturation S_w was defined as in equation 17 by Ni, Datta and Torrance (1999)

$$S_w = \frac{c_w}{\rho_w \phi} \qquad (17)$$

where c is mass concentration (kg/m^3) and thus, equation 15 can be simplified to equation 18.

$$\vec{n}_w = \underbrace{-\rho_w \frac{k_w}{\mu_w} \nabla P}_{\substack{\text{flux of water} \\ \text{due to pressure}}} - \underbrace{D_w \nabla c_w}_{\substack{\text{concentration} \\ \text{driven water flux}}} - \underbrace{D_T \nabla T}_{\substack{\text{temperature} \\ \text{driven water flux}}} \qquad (18)$$

To obtain effective heat transfer and predictable heat transfer coefficients between heating medium and fish, several processors choose to process the vacuum packed portions in an autoclave under an overpressure high enough to prevent formation of vapour and to assure good contact between the pouch and the product. Under such conditions the capillary diffusivity of water, D_w is given by equation 19.

$$D_w = -\rho_w^2 g \frac{k_w}{\mu_w} \frac{\partial h}{\partial c_w} \qquad (19)$$

where g is gravity (9.81 m/s^2) and h is enthalpy (J/kg).

To measure or determine the constants used in these equations is outside the scope of this thesis. Still, the principals of the water flux out of a vacuum packed portion of fish from the three terms in equation 18 can be discussed in light of the observed cook loss:

(1) Provided the density, permeability and viscosity of water is constant (true under isothermal conditions), the water flux is increasing linearly with increasing pressure. However, the pressure gradient is not increasing linearly since it is a result of
   (a) muscle shrinkage caused by several steps of thermal protein denaturation, i.e. a function of temperature and time
   (b) thermal expansion of water and gases in the fish meat, i.e. a function of temperature

   Indeed, the observed cook loss is increasing with temperature as proposed by equation 18 but increasing much faster at the end of the major protein transitions.

(2) Water expelled will surround the fish and a concentration gradient will build up and contradict the expulsion of water. Depending on the time/temperature history and the condition of the fish, proteins

will be dissolved in the expelled water and decrease the concentration gradient. Water will also be released from proteins and increase the concentration gradient in the opposite direction. The concentration gradient term may explain the observed cook loss at low temperatures or during heating to a higher temperature.

(3) The temperature gradient is given by the heat equation and will be log-linearly decreasing from a maximum when heating is started and approach zero as the temperature in the fish is evening out. This is consistent with the observation of little or no increase in cook loss over time at constant temperature after the protein denaturation, which is completed within a few minutes at constant temperature.

The three terms in equation 18 could in principle fit well with the observed data but should incorporate the protein denaturation effects. It has previously been shown that heat induced structural changes (Ofstad et al., 1993) and protein denaturation (II) is irreversible. During cooling the new configuration of the fish meat established during heating will remain unchanged. Only a small amount of the liquid expelled during heating will be absorbed by the fish during cooling and it is not expected to cause any swelling but rather follow equation 18. Further research on protein denaturation would indeed help in establishing this model, but it is possible this could be studied indirectly by measuring shrinkage of fish meat during heating. Studies of salmon heated at temperatures in the range 100°C to 130°C has shown that the cook loss is relatively constant after 30 min heating time and this plateau level was a function of the temperature and correlated well with area shrinkage of a disk shaped sample (Kong et al., 2007b). This rapid increase before reaching the plateau level seems to be the same behaviour as seen from the cod at temperatures below 100°C.

2.1.2.2 Loss of WHC

The results for WHC screened by isothermal heating for 0 to 60 min at temperatures in the range of 20ºC to 95ºC are shown in figure 7. Several time and temperature combinations resulted in excessive loss of WHC, e.g. 45°C for 20 min or 90°C for 50 min gave a WHC less than 50%. These heat loads may be associated with a high drip loss during distribution and storage and loss of juiciness. On the other hand, WHC was observed in the range 66-70% when the cod was subjected to an intermediate heat load, e.g. 30 min at 60°C. This intermediate heat load area is shown in dark green in the middle of figure 7. Only raw fish (i.e. heated to less than 35°C or for less than 2 min) resulted in higher WHC.

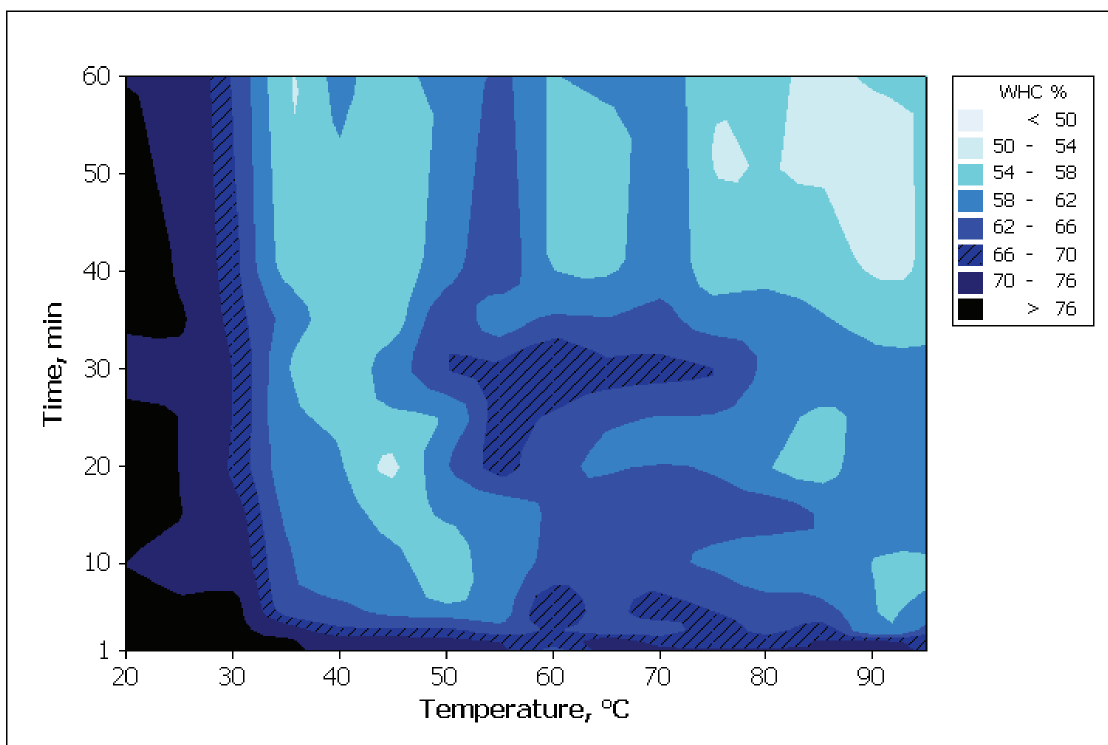

Figure 7. Water holding capacity (WHC) at cooking times from 1 min to 60 min and heat treatment temperature from 20 ºC to 95°C (adopted from V).

Cook loss and reduction in WHC as a result of heat treatment of coarsely ground muscle from farmed Atlantic cod has been quantified. As expected, increasing temperature and increasing processing time

result in loss of WHC. However, within a limited range of processing time and temperatures, there was a local maximum in WHC coincidencing with the range for inactivation of *L. monocytogenes*. In this range it is possible to keep WHC above 66 % and the cook loss below 5.6%. Therefore pasteurisation of cod should be done approximately at 68°C ± 4°C within a processing time in the approximate range 25 to 35 min.

Both loss of WHC and cook loss origins from a combination of physical reasons (i.e. concentration and temperature gradients) and protein denaturation. Protein denaturation results in changes in the internal structure of the fish muscle and the capillaries therein. Shrinkage and mechanical expulsion of juice is another effect related to protein denaturation.

2.1.2.3 Texture and colour changes

It was expected that measurements of texture could be used to validate the results from the model system as texture is closely related to water content and protein denaturation (Dunajski, 1979). A TPA (Texture Profile Analysis) on cooked material did not result in reproducible results because the angle of the myotoma is impossible to standardize after cooking. Texture measurements on single, intact myotoma were therefore the used to include direct measurements of texture (V). The changes in texture with increasing heat load are expected to reflect the overall denaturation of individual proteins. A TPA test was therefore conducted for myotoma samples with different probes and settings. The TPA test consists of compressing a small piece of food two times in a way that imitates the action of the jaw and extracting from the resulting force-time curve a number of textural parameters that have been found to correlate well with sensory evaluation (Bourne, 1978; Bourne, 1992). The primary parameters of hardness, cohesiveness, springiness (elasticity), and adhesiveness, and the secondary (or derived) parameters of fracturability (brittleness), chewiness and gumminess can be determined by the TPA (Szczesniac, A. S. 1966).

The hardness of samples heat treated at different temperatures was not significantly different from each other (V). All cooked sample s were significantly harder than raw samples and there was also a tendency to

increasing hardness with increasing heat load. Further measurements would be needed to verify a possible correlation between texture and WHC.

When heated, the opaque cod muscle changes to white. This happens while heating from 30 °C to 40°C. At temperatures from 70 °C to 90 °C there was no significant change in colour. The whiteness score of samples treated at temperatures above 60°C was higher than for all samples treated at lower temperatures.

2.1.3. Protein denaturation

Protein denaturation and the resulting shrinkage of muscle fibrils was expected to be the main reason for both water loss and textural changes in fish during thermal processing (Ofstad et al., 1993). Therefore, protein denaturation studies were expected to aid to reveal the mechanisms behind these major quality changes. The aim of the following experiments was to quantify protein denaturation of the most important proteins, estimate kinetic parameters and investigate possible correlations with changes in WHC and texture.

Protein denaturation studies on minced fish were carried out using differential scanning calorimetry (DSC). Ground and frozen cod was hermetically sealed in crucibles and analysed using a differential scanning calorimeter (DSC7, Perkin-Elmer). A linear heating rate (10°C/min) was applied.

Figure 8 shows the results from the scan of a typical raw sample (II). The energy transitions were concentrated in 5 distinct peaks which was overlapping each other. These peaks could through literature (Howell et al., 1991) be related to specific proteins but it was not attempted to separate all of the peaks. However, peak 5 which represent denaturation of actin could be separated from the others by pre-heating the samples before the scan. The total denaturation enthalpy for all proteins could also be determined for samples processed before the scanning in the calorimeter. For peak 5 it showed possible to determine kinetic parameters for a log-linear model describing the residual reaction enthalpy.

Denaturation of cod muscle proteins has been compared to cook loss and loss of water holding capacity and it has been shown that the protein denaturation occurs in a lower temperature range (35-66ºC) than the appearance of major cook loss (above 80ºC) when cod muscle is heated (II). Other mechanisms for release of water than protein denaturation should therefore also be considered but as this is in addition to liquid loss due to denaturation of several proteins it does not fit into a first order model and cannot easily be quantified by a C-value. However, kinetic parameters for a first order inactivation of

separate proteins are available, i.e. a D- and z-value for actin have been presented (II) and may perhaps be used for C-value calculations if it can be related to sensory acceptance of a cooked appearance.
The peak maximum temperatures for myosin were found at 38.4°C and 44.1°C. However, myosin denaturation starts at about 28°C and continues to about 50°C and during this process the myosin chains will contract, split and form a new and more open structure with reduced ability to bind water. From 50°C to 72°C sarcoplasmic proteins are denatured, and the muscle is shrinking the capillaries that were opened partially closes. Thus, the water holding capacity is increasing again but this development is reversed by denaturation of actin which starts already at about 58°C. At this temperature the denaturation of actin is a slow process and even at 68°C it will take about 13 min to denature 90% of the present actin and the peak maximum is reached at 76.1°C.

A 6 log inactivation of *C. botulinum* spores would require a heat treatment equivalent to 10 min at 90°C. Such a heat load, will denature all the major proteins. This will result in a high cook loss and a low WHC which cannot be avoided by optimisation of the heat process alone. To retain the juiciness characteristic for a fresh made meal of heat treated cod during a refrigerated shelf life of more than 3 weeks, other measures will have to be taken. Such measures may be addition of additives (e.g. salt and phosphates) or by using hurdle technology. Typical hurdles for inhibiting growth of *C. botulinum* is use of low pH (no growth below pH of 4.6) or temperature (no growth below 3 °C).

The specific heat of protein folding, ΔH_{D-N} has been discussed towards changes in entropy, ΔS_{D-N} and free energy of unfolding ΔG_{D-N} (Fersht, 1998), which are related through equation 20.

$$\Delta G_{D-N} = \Delta H_{D-N} - T\Delta S_{D-N} \qquad (20)$$

These changes in energy are well worth further studies. Folding kinetics of proteins can now be measured by single-molecule force spectroscopy assisted by atomic force microscopy (Jollymore & Li, 2010). Such basic research may give a lot more detailed information about the mechanisms of thermal protein denaturation. In vitro studies will, however, still be necessary for the overall picture due to the complexity of the fish muscle.

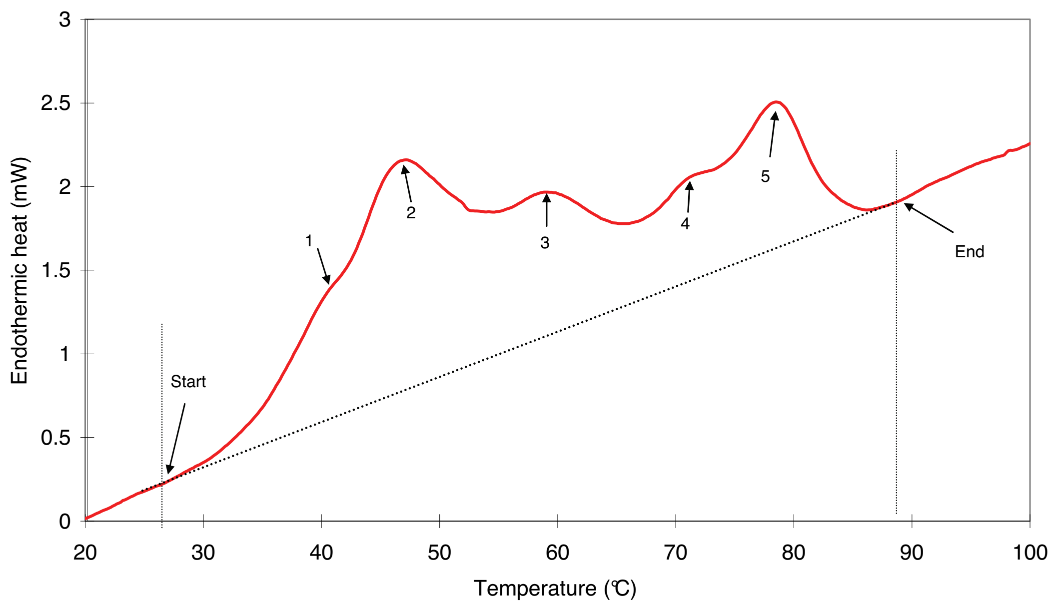

Figure 8. Typical DSC-thermogram of untreated cod muscle. Numbers indicate the peaks of protein denaturation (reworked from II).

2.2. Food safety and shelf life

2.2.1 Method for determining heat load on enzymes and microorganisms

Different enzymatic time-temperature integrators (both intrinsic and extrinsic) for the quantification of thermal processes in terms of food safety are extensively described by Hendrickx et al. (1995) and Van Loey et al. (2004).

Acid phospatase (ACP, EC 3.1.3.2) has been suggested as an intrinsic TTI for different meat products (Kormendy et al., 1992; Orta-Ramirez et al., 1997). Kuda et al. (2004) have measured the inactivation of ACP in different fish species during heat treatment but no kinetic were generated. Therefore it was investigated by a kinetic study if acid phosphatase (ACP) could be used as an intrinsic biological marker for heat induced quality changes or *L. monocytogenes* inactivation.

2.2.1.1. ACP in extracts (paper III)

Analyses on the thermal inactivation of ACP in cod were performed on samples from a homogenous batch of crude extract prepared from fresh cod in order to examine the kinetics of acid phosphatase inactivation. The extracts were prepared by adding 1 % Triton X-100 (1:9, w:w) to minced cod loins before blending and centrifugation. After filling the supernatant in capillary tubes sealed by rubber stoppers, the extract (0.5 ml) was heated in a water bath. Temperature history and uniformity was recorded to be able to correct for heating lags and cooling lags. This was of particular importance when analysing inactivation at short time intervals at temperatures above 60^0C. The inactivation study was then performed at 10 measuring points for each temperature tested in order to reach a two log reduction of the enzyme activity. After heat treatment the extracts were applied to a multiwell plate and added a mixture of magnesium chloride and PNP-substrate (4-nitrophenyl phosphate di-sodiumsalt hexahydrate) dissolved in sodium acetate. The multiwell plates were

incubated for 30 min at 37°C and the enzyme-substrate reaction was quenched by addition of NaOH. The ACP activity was measured in a spectrophotometer as the absorbance of *p*-nitrophenol (405 nm) released from the substrate.

Kinetic parameters for heat inactivation of ACP activity in extracts were estimated from the data to $D_{60°C} = 6.78 \pm 0.10$ min and $z = 6.37 \pm 0.09$ °C in the range of 55-67.5 °C for 2 to 60 min. The log-linear inactivation pattern was promising and it was concluded that the methodology could be used fro further experiments. With respect to product shelf life, ACP could possibly be used as a TTI for enzyme inactivation. However, it was also indicated that the heat resistance of ACP in extracts was too low to serve as a general TTI for *L. monocytogenes* inactivation and a more detailed study over a wider temperature range was needed.

2.2.1.2. Kinetics of enzyme (ACP) inactivation *in situ* (paper IV)

Inactivation studies (time-temperature) of ACP were performed on whole tissue samples as well as extracts, using the same loading and heat processing procedures as for WHC analysis (sample cups in steel). The inactivation was studied in the range 54-70°C for extracts and in the range 56-68°C for 15 s to 25.5 min for muscle. The thermal inactivation studies were carried out in a water bath followed by cooling and muscle extraction for spectrophotometric ACP analysis at standard conditions (pH optimum, 5.5 – 37^{0}C – 30-60 min). The practical application of the ACP as a TTI was validated based on residual ACP activity measurements from homogenously heated and cooled portions of cod. The results are shown in figure 9.

The use of relatively short heating times and low temperatures relative to the subsequent study (III) revealed a biphasic inactivation of ACP *in situ* which could not be demonstrated for ACP in heat treated extracts with the established methodology. This was an unexpected limitation for the method. However, for heating times longer than 100 s the kinetic parameters could be established to $D_{60°C} = 6.78 \pm 0.10$

min and z = 6.37 ± 0.09 °C. Thus, the data support a 4 log inactivation of *L. monocytogenes* at 70°C and up to a 6 log inactivation at 60°C.

Conduction heating of fish portions by conventional heating methods (e.g. in ovens or autoclaves) typically takes a lot more than 100 s, and the biphasic behaviour is therefore not of major concern. Inductive heating (e.g. by microwaves) could possibly reach the desired temperature in shorter times, but cooling would still have to be done by conduction. ACP could therefore be used as a TTI for a large range of industrial applications.

The gender and seasonal variation did not affect the level of ACP in cod muscle. Freezing of the raw material caused a short initial drop in ACP activity but was stable during 24 months of storage at -80°C. Brining of the muscle with different levels of salt and phosphates did not affect the level of ACP or its heat resistance.

The ability of determining the microbial inactivation of a thermal process is crucial to avoid over-processing. Kinetics of thermal ACP inactivation has been determined and is now available as an intrinsic biological indicator for post process determination of the heat load on cod.

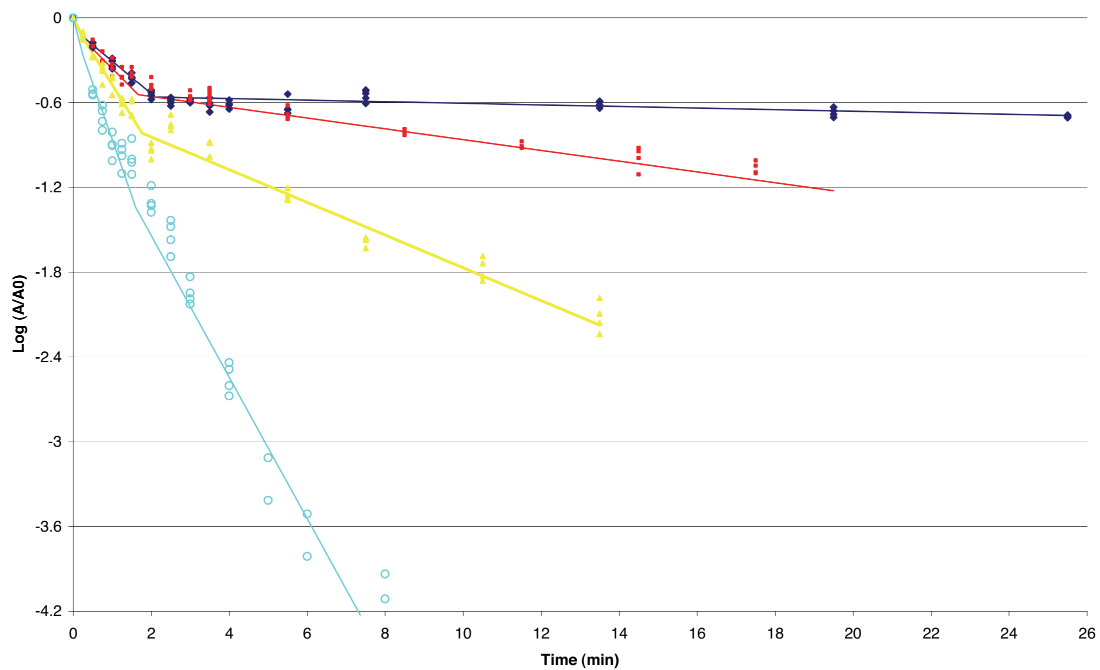

Figure 9. The residual ACP activity versus isothermal heating time at temperatures 56ºC (dots •), 60ºC (squares ■), 64ºC (open circles ⊙) and 68ºC (triangles ▲), of muscle with regression lines (adopted from IV)

3. Conclusion

In this thesis, optimisation of thermal processing of convenience products of vacuum packaged farmed Atlantic cod (*Gadus morhua*) has been discussed. The limits of existing knowledge in the field have been revealed and adequate methods and need for new methods for the experimental work of the project have been identified.

The microbial constraints have been summarised. It has been concluded that a 6 log inactivation of either *L. monocytogenes* or non-proteolytic *C. botulinum* is required for refrigerated heat processed cod, depending on the required shelf life.

It has been the intention to determine the kinetics of important quality attributes and combine the kinetic models with a thermal model and models of microbial inactivation into a multiphysics model. During this work the following is achieved:

- A method for determining cook loss and WHC under isothermal conditions has been developed, including the possibility of determining texture and colour of samples isothermally heated. The method has been made commercially available.
- Cook loss and WHC changes during thermal processing in the range 20 °C to 95 °C has been quantified. It has been shown that a local maximum for WHC coincidences with the minimum heat load required for inactivation of *L. monocytogenes* at time/temperature combinations around 70 °C for 30 min.
- Thermal protein denaturation has been quantified and the kinetics of actin denaturation has been determined
- The ability of determining the microbial inactivation of a thermal process is crucial to avoid over-processing. Kinetics of thermal ACP inactivation has been determined and can be used for post process determination of the heat load on cod.
- With respect to texture and colour it has been shown that the major changes occur after relatively short processing times at temperatures far below the required heat load for obtaining safe products.

In conclusion, several tools needed for optimisation of the thermal processing has been developed and the first steps towards a complete numerical model of quality changes during thermal processing of cod have been taken.

4. Further studies

Further studies should pursue a mathematical model for loss of water that could show to be useful if combined with models for inactivation of pathogens and a heat transfer model. One way to collect the necessary information could be to determine the kinetics of fish muscle shrinkage. Next step would be to implement the optimisation model in product development for actual industrial products. The methods developed for measurement of cook loss and WHC in this project could show to be very useful for other fish species as well as cod. During development of commercial products the models should be evaluated, e.g. by sensorial methods. Finally, the inactivation of enzymes and microorganisms should be evaluated. The use of ACP as intrinsic biological indicator could be evaluated for processes that are challenging to validate in other ways, e.g. microwave heating.

The following presents a suggestion for building a numerical model for optimisation of the thermal processing of vacuum packaged cod portions.

4.1. Optimisation of thermal processing

A heat transfer model for processing vacuum packaged fish portions in steam or water bath with both convective and conductive heat transfer was developed for designing an optimal thermal process with respect to the quality of cod muscle. This can provide knowledge crucial for the introduction of commercial thermal processing of farmed cod and contributes to the knowledge of fish processing in general.

4.1.1 Method for model construction

In a commercial food chain it is expected that the portions are heat treated and cooled by a commercial producer before wholesale and then reheated by the consumer. Thus, experiments on both single and repeated heating should be made. The analysis is limited to sensorial testing and measurement of cook loss and WHC. Texture analysis may be investigated further, but as the heat load throughout the portion is far from homogenous, it cannot be expected to obtain

consistent results. Practical difficulties may also make texture measurement of cooked portions unfeasible. Texture can be analysed sensorial if instrumental analysis fails to give consistent results.

A combined convection and conduction finite element model has been built on a commercial available platform, Comsol Multiphysics. This software will make it feasible to implement kinetic models for quality attributes and design thermal processes that can be evaluated by experiments. Conductive heat transfer has been modelled based on the Fourier equation and, as a starting point; the convective heat transfer coefficient at the boundaries of the fish may be set to a constant value. Convective heat transfer may also be calculated by a coupled heat transfer and incompressible flow model utilising the Navier-Stokes equations for fluid flow. It is expected that the final model can be used to calculate the time/temperature history at any location of the fish portion during processing and both the resulting pasteurisation and cook values. The thermal model can be validated using single spot temperature measurement with thermocouples and pictures of cross sections of heated portions taken with a thermal camera.

Specific heat capacity (c_p) of minced fish should be further investigated by DSC. Density of cod (ρ) is known from Waterman (2001) but should be evaluated experimentally for farmed cod. Heat conductivity is known from literature.

As already mentioned, a numerical model was made in Comsol Multiphysics (version 3.5) for a vacuum packaged portion of cod muscle. The dimensions of the cod portion was 100*60*24 mm (length, width, height) and the weight was 127g (figure 10). Thermal conductivity was measured to 0.42 ± 0.03 W / (m K) (KD-2 conductivity meter). The model (4) by Sweat (1995) suggests a thermal conductivity of 0.49 W/m K for a cod composed as in our experiments (II), which is in the same order of magnitude as measured (II).

Specific heat capacity was 3650 kJ/(kg K) and density 1055 kg/m^3 (II). The heat equation for conduction was used for calculation of the heat transfer in the fish portion,

$$\rho\, C_P \frac{\partial T}{\partial t} - \nabla \cdot (K\, \nabla T) = Q \qquad (21)$$

where T is temperature (°C) in the fish and t is time (s). As boundary conditions, convective heat transfer coefficient for heat transfer from steam/water to fish portion was set to 10 000 W/(m^{2}K) for the heating period and to 4000 W /(m^{2}K) for cooling of the fish, which are typical values for heating and cooling in a raining water autoclave. A grid of elements was automatically generated as shown in figure 10. The thermal model was evaluated by measurement of core temperature in 8 fish portions with thermocouples and E-val flex datalogger (Ellab, Copenhagen, Denmark).

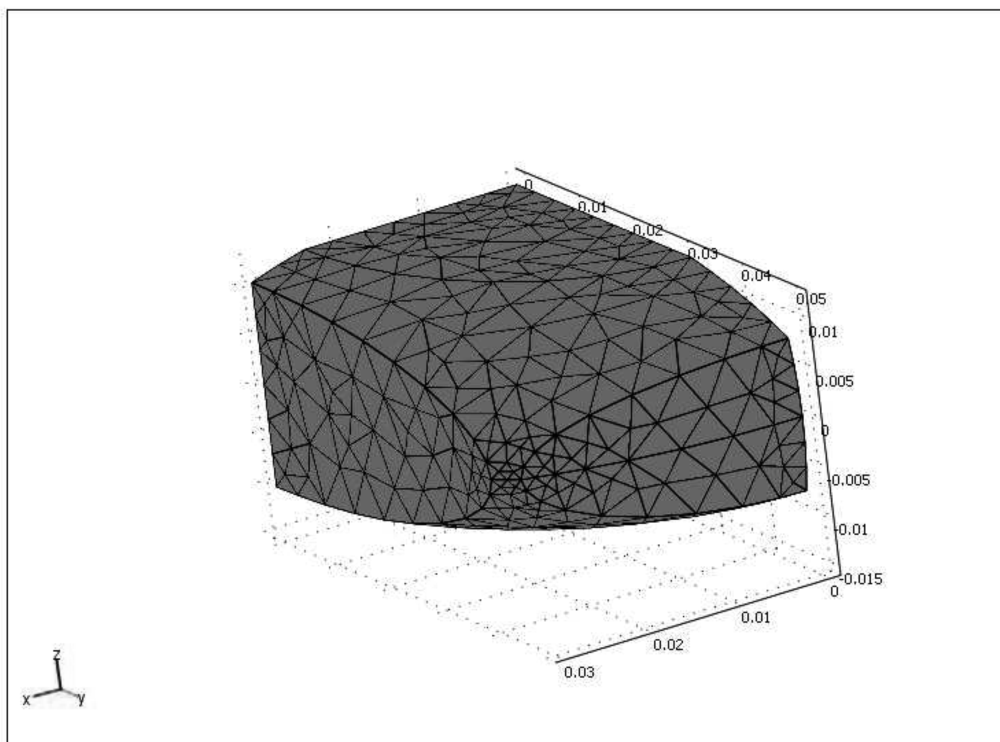

Figure 10. Geometry of ¼ portion of fish. Axis labels in m

4.1.2 Expected outcome of a numerical model

The temperature prediction of the thermal model showed to fit well to the measured temperatures as shown in figure 11. Increasing the convective hest transfer coefficient towards infinity did not change this picture, thus the model should be valid for heating in both steam and water.

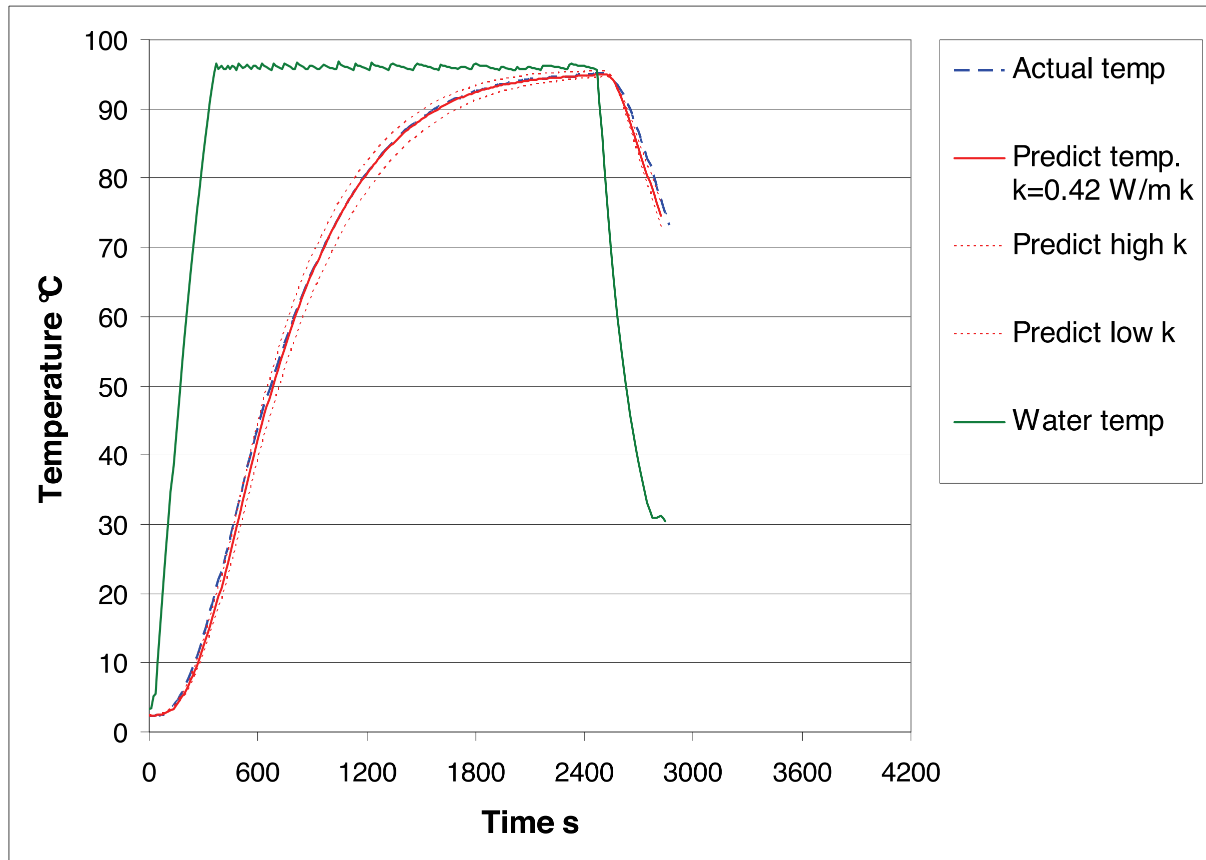

Figure 11. Ambient temperature (green) and average core temperature in vacuum packaged portion of cod measured by thermocouples (blue, dotted line) compared to core temperature predicted by numerical model (red line). Dotted red lines is core temperature predicted for thermal conductivity of ± one standard deviation.

Based on commonly accepted safety criteria for heat inactivation of *L. monocytogenes* and the thermal model, the lethality distribution in the fish portion was calculated for a process of 10 min heating to 75 °C and kept there for 30 min. A 6 log inactivation can be represented as a pasteurisation value (lethality, u in s) of 120 s when reference

temperature is 70°C. The total lethality achieved is shown in figure 12. The pasteurisation value was calculated by equation 22,

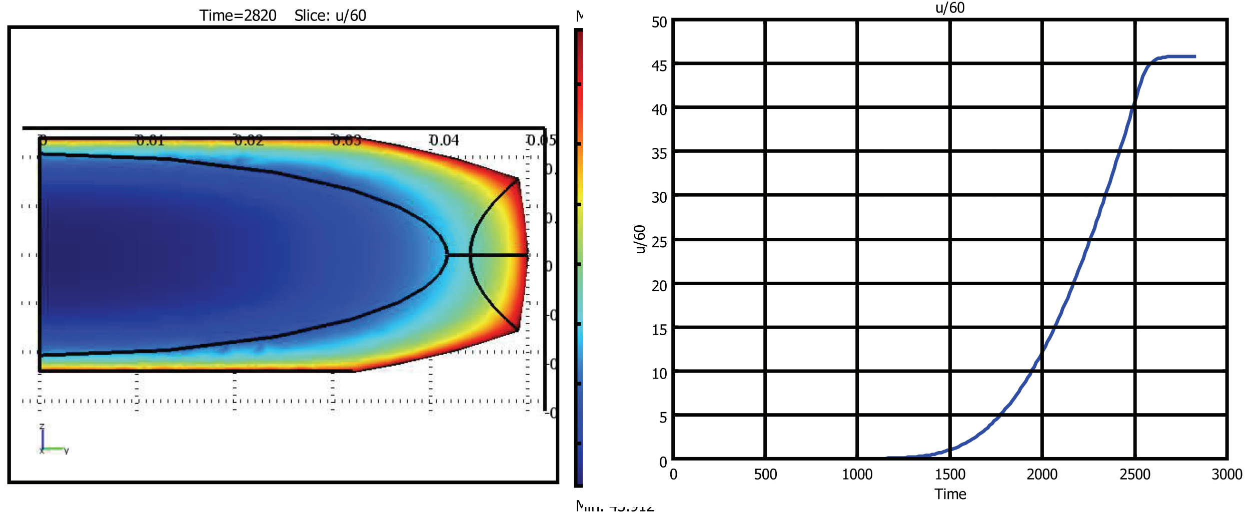

Figure 12. Lethality distribution in fish portion (left) and lethality (min) in core of product (right) as function of time (s)

$$\frac{\partial u}{\partial t} = 10^{\frac{T - T_{ref}}{z}} \qquad (22)$$

where T is the temperature in the fish (°C), T_{ref} is the reference temperature for inactivation of the microorganism and z is the temperature increase necessary to obtain a tenfold decrease of the D-value (°C).

A numerical model for optimising thermal processing of cod muscle may be further developed with the ability to

- Predict the temperature in the model fish product
- Calculate inactivation of bacteria
- Calculate protein denaturation
- Predict quality changes under different processing conditions and processing parameters.

Sensory analysis may be used to confirm the sensory quality of raw material and thermally processed portions. The results of the modelling approach described above may be used for optimizing the

thermal processing of cod and samples may be produced on pilot scale. Sensory evaluation of the products may be used to evaluate the samples. Different sensory methods are available. For sensory evaluation of colour, odour and flavour, the Torry freshness scheme may be used (Shewan et al., 1953) or the quality index method (QIM) (Bonilla et al., 2007). The Quantitative Descriptive Analysis Scheme (QDA) is feasible for evaluation of texture (Stone & Sidel, 1985). A sensory vocabulary is also developed for cooked samples of cod and has shown its ability to differentiate between different cod products (Sveinsdottir et al., 2010).

Finally, a consumer study may be performed and after further development, new, healthy and convenient cod products may be presented in the market. It is expected that the methodology presented in this thesis may be used for investigating and modelling optimal processing conditions may be utilised for other fish species as well as for other variants of cod products (e.g. salted/brined cod).

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A method for characterising cook loss and water holding capacity in heat treated cod (*Gadus morhua*) muscle

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Abstract

The currently available methods for measuring water holding capacity (WHC) have been developed for raw material characterisation and have been applied to processed (cooked) muscle samples. The intrinsic shortcoming of the methods is that they do not allow rapid and uniform heating, which make them unsuitable for investigating the kinetics of cook loss and WHC. A new method and associated equipment (sample cups) for rapid and uniform heating that allows studying cook loss, water holding capacity and texture of fish samples was developed. After heating and cooling of samples in the sample cup it is feasible to gravimetrically determine the cook loss with a standard deviation within 30% of the total cook loss for minced samples. Both intact and coarsely minced muscle of farmed cod was used to investigate the abilities and limitations of the method with respect to WHC.
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Keywords: Thermal processing; Cook loss; Water holding capacity; Cod; *Gadus morhua*; Fish

1. Introduction

Minimally processed convenience fish products are a growing segment of the prepared food market. Farmed species are of special interest as seasonal variations can be avoided and a more reproducible quality can be achieved. With the introduction of farmed cod there is also a need for understanding changes in the product during processing in order to optimise the thermal processing. Farming of cod is currently a fast growing industry and has received much attention (Hemre, Karlsen, Mangor-Jensen, & Rosenlund, 2003; Jobling, 1988; Lauritzsen, Akse, Gundersen, & Olsen, 2004; Lauritzsen, Akse, Johansen et al., 2004; Morais, Bell, Robertson, Roy, & Morris, 2001; Puvanendran & Brown, 1999; Tilseth, 1990).

The water holding capacity (WHC) of muscle foods is important for several reasons and depends on heat-induced structural changes, sarcomere length, pH, ionic strength, osmotic pressure and state of *rigor mortis* (Ofstad, 1995). To the consumer the most obvious quality parameters are flaking (whether the fish is falling apart on the plate), texture, tenderness and juiciness. These parameters are related to the WHC as they all depend on heat denaturation of proteins. WHC has even been used as measure for quality (Rustad, 1992) and for characterising protein denaturation. For a product heat treated in a vacuum package, accumulated exudate is also unattractive to the consumer and is yet another reason for the food processing industry to reduce loss of water during thermal processing.

Aitken and Connell (1979) reviewed the effects of heating on fish and reported that cook losses varied greatly with the fish species, the method of heating and the heating regime (i.e., sterilization, pasteurization, etc.). There are, however, few publications discussing the effects of heat on cod.

WHC of cod during heating has been studied by Ofstad, Kidman, Myklebust, and Hermansson (1993), who showed that the main structural changes appeared in the connective tissue at low temperatures (<40 °C) and concluded that

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water loss at these temperatures is mainly due to denaturation and melting of collagen. Ofstad et al. (1993) used a heating rate of 1 °C/min to the required temperature and 10 min holding time, placed the solid material on a net in a sample holder and centrifuged at 210*g* for 15 min. They found that maximum water loss was attained when the muscle cell shrank due to denaturation of myosin and the extra-cellular spaces were widened, and the centrifugation loss decreased as a function of temperature when extra-cellular, granulated material became visible. Ofstad et al. (1993) found a maximum centrifugation loss between 40 °C and 60 °C before it decreased.

Several methods can be used for measuring WHC, the first one reported was developed by Childs and Baldelli in 1934 (Trout, 1988). For fish products, a low speed centrifugation is commonly used and several variants of the method can be found. The fish muscle is centrifuged at constant speed for a desired time and the liquid loss is measured as the amount of liquid that passes through a filter in the bottom of the sample cup. The water holding capacity can then be determined by either the weight of the released liquid or by weight loss of the sample. The different methods give only relative WHC values. Results in terms of absolute values from different methods can therefore not be compared (Trout, 1988). For centrifugation in the range 200–800*g*, there are some changes in the microstructure of the sample. The results are also strongly dependent on the processing and the fish raw material characteristics.

Most of the publications on the topic have focused on WHC in raw material that has been minced or ground. The existing systems in use have not focused on the effects of heating with exception of the thesis of Ofstad (1995). Existing equipment has mainly been made in plastic materials with a wall thickness that will isolate the sample and if a heating block is used it would certainly result in an uneven temperature distribution in the sample. For optimisation of a thermal process there is a need for kinetic data for the change in WHC as function of heat load, i.e., the combination of time and temperature during processing.

The main objective of this work was to develop a new method for characterising the effects of heat-treatment on cook loss and WHC of whole and comminuted muscle tissue of farmed Atlantic cod (*Gadus morhua*). Compared to earlier methods, we aimed at a method and associated equipment designed for rapid and homogenous heating of fish samples to a constant temperature (isotherm) and with the possibility of measuring texture and WHC. In a second phase, we evaluated the feasibility of the method to detect differences between samples cooked at different temperatures for both frozen and fresh material.

2. Materials and methods

The work was developed based on three series of experiments:

1. A preliminary experiment on minced fish to determine the standard deviation of the method
2. A second experiment to investigate the heating rate and uniformity of the method and the difference between whole and minced fish material
3. A third experiment using both frozen and fresh whole material (myotoma) at different temperatures to determine the feasibility of the method.

The same sample cup and a heating bath was used in all three experiments as described below.

2.1. Sample preparation and sample cups common for all three experiments

WHC was measured in both ground cod (experiment 1) and whole samples cut from the loin by a scalpel and a punching tool (experiments 2 and 3). Loin from filleted and de-skinned fish was used for all samples. Each segment (myotoma) was cut from the fillet by a scalpel and cut to a sample of diameter 31 mm. Two or three myotomes were combined to obtain a total weight of 5 g and a height of 6 mm. Four parallels from each variant and four variants per fish were used. The samples were statistically randomized by Minitab R14.13 (Minitab, USA).

The samples were transferred to sample cups (Patent No. 321375 B1), a sample cup in stainless steel, diameter 31 mm with filter 0.213 mm mesh (Norconserv, Stavanger, Norway). The sample cup is designed for analysis of cook loss, WHC and texture of cooked material but is also convenient for other analysis as for instance colour. As shown in Fig. 1 the temperature in the sample may be recorded by a probe tread through a specially designed lid. Fibre optic temperature sensors were used to record the temperature.

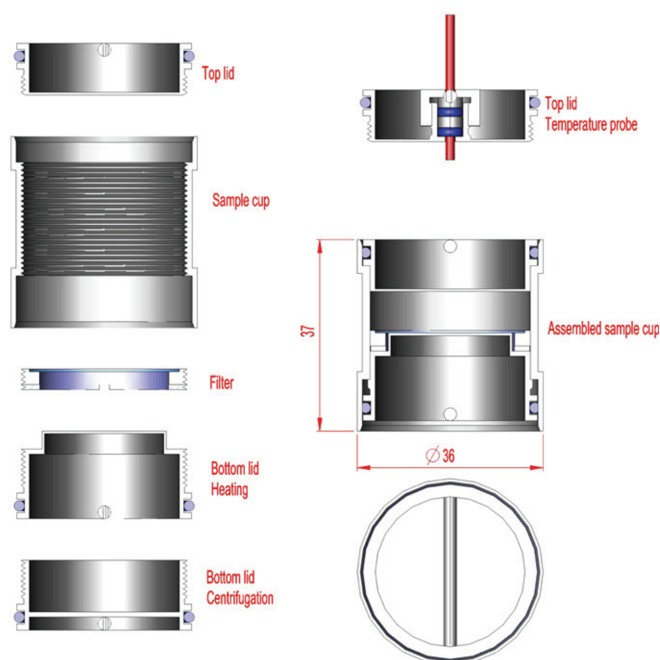

Fig. 1. Sample cup developed for the experiments (measures are in mm).

The thickness of the circular sheets surrounding the sample is 0.5 mm and provides negligible insulation to the food material.

In all heating experiments the lid was mounted first and the cup was weighed. Then the fish was placed inside the cup in good contact with the inside of the lid and weighed. The filter was then mounted inside the cup and screwed until the filter was in contact with the fish. The bottom lid was mounted until it came to a lock with the filter. The sample cup containing the sample was then immersed in the heating bath, heated for the required time at the required temperature, and subsequently cooled in ice water. Before centrifugation, the bottom lid was removed, the cook loss determined gravimetrically and the bottom lid for centrifugation was inserted.

For measurement of texture (experiment 3) the bottom lid was not removed after heating and cooling. Instead the top lid was removed and the bottom lid placed on a holder designed to fit into the sample cup. In this way it was possible to maintain a constant height from the holder to the sample which was necessary for measurement of texture.

2.2. Assessment of heat transfer from heating medium to sample

Temperature was measured in the sample and in the bath both for immersion in oil and in water. Coarsely minced and intact muscle was placed in the sample cup and heated in circulating silicon oil; 60 °C, 75 °C and 90 °C for 10 min. Temperature uniformity in the oil was measured by 12 temperature sensors (Tracksense Pro, Ellab, Denmark). The temperature in the oil at any point in the oil bath was within ±0.5 °C of the average and the stability of the bath during the heating time of 10 min was within ±0.1 °C. Oil circulation speed was measured by a flow meter (Dostman P600, Dostman electronic GmbH, Germany) to an average of 0.10 m/s and used as input together with the temperatures in the oil in a 3D finite element method model programmed in Comsol Multiphysics 3.2 (Comsol AB, Lund, Sweden). The model was solved in two steps. In the first step, the built in module of the software for stationary incompressible flow was calculated by the Navier–Stokes equations to find the heat transfer coefficient from the heating medium flowing over the surfaces of the sample cup. The heat transfer coefficient was used as boundary condition for a transient conduction model of the sample cup with the fish sample. After heating for 10 min, the samples were cooled in circulating ice water at 0 °C for minimum 90 s to an end temperature in the range 0–1.8 °C.

2.3. Water content and cook loss

Cook loss was measured as weight of sample before and after draining for 30 s. The surface of the cup lid was dried with a cloth. The dry matter content of fish muscle was determined gravimetrically after drying at 105 °C for 16 h (NMKL 23, 1991). The dry matter content of cook loss was determined the same way as for the fish.

2.4. Water holding capacity (WHC)

The analysis of WHC was based on the method described by Børresen (1980), but the centrifugation speed and time were modified and the new sample cup was used. Three different centrifugation speeds used in earlier methods were included; 173*g*, 326*g* and 528*g* for 15 min each. Weight loss was then registered. Different heating temperatures were used to evaluate if the method could be used for describing the kinetics of WHC in cod during thermal processing.

The standard deviation of the proposed method was tested with a homogenously minced fish. In this test, eight samples were heated at 90 °C for 10 min, cooled and centrifuged together with eight raw samples at 528*g* for 15 min.

Liquid loss was measured for all the samples as the amount of liquid that passes through a filter in the bottom of the sample cup. The water holding capacity could then be determined by the weight of the liquid released. Fig. 2 shows the composition of the samples divided into water and dry material. The initial weight of a raw sample m_0 is equal to the sum of the initial water content of raw material, V_0, and dry material, D_0. The weight of the exudates (the liquid separated from the sample during centrifugation) is named ΔV_0 for raw samples. The solid contents of the exudates are regarded as negligible in these calculations.

WHC of raw samples were calculated as percentage remaining water of initial water in sample

$$\mathrm{WHC} = \frac{W_0 - \Delta W}{W_0} \cdot 100\%$$

where

$$W_0 = \frac{V_0}{V_0 + D_0} \cdot 100 \quad \text{and} \quad \Delta W = \frac{\Delta V_0}{V_0 + D_0} \cdot 100$$

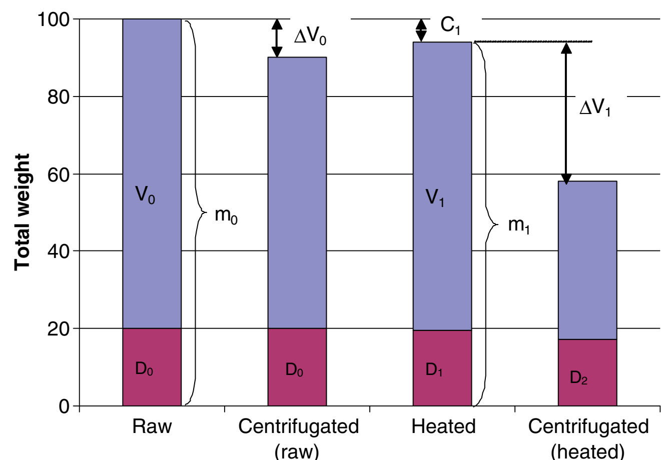

Fig. 2. Weight of sample before (m_0) and after heating (m_1) split in dry materiel (D) and water content (V). Cook loss is shown as C_1 and centrifugation losses as ΔV_0 and ΔV_1 for raw and cooked materiel, respectively.

when the samples were cooked, liquid was expelled from the fish as cook loss, C_1. This cook loss consists of water, dissolved proteins, ash, salt and fat. The remaining dry material in the sample, D_1, will be in the range 0–6% smaller than D_0. During centrifugation additional dry material is removed from the sample together with the centrifugation loss and the remaining dry material, D_2, will be significantly lower than D_0.

For cooked material a WHC_1 can be defined

$$WHC_1 = \frac{W_1 - \Delta W_1}{W_1} \cdot 100\%$$

where

$$W_1 = \frac{V_1}{V_1 + D_1} \cdot 100 \quad \text{and} \quad \Delta W_1 = \frac{\Delta V_1}{V_1 + D_1} \cdot 100$$

Thus,

$$WHC_1 = \frac{V_1 - \Delta V_1}{V_1} \cdot 100\%$$

By using this calculation method the WHC will describe the ability of the cooked material to withhold the water during centrifugation. Dry matter lost during the centrifugation step is included in the calculation. This is common practice as in practical terms it represents a real loss. However, the dry matter content may be determined either directly from the exudates or via the dry matter content of the centrifuged fish material and subtracted from the rest of the centrifugation loss. As the water expelled during cooking (before centrifugation) is not taken into account, the method will not describe neither the water loss nor the protein denaturation very well. A suggestion to improve the method is to calculate the WHC on the basis of the water content of the raw material and including the cook loss in a total WHC (WHC_{TOT}) which can be calculated by

$$WHC_{TOT} = \frac{W_0 - \Delta W_{TOT}}{W_0} \cdot 100\%$$

where

$$\Delta W_{TOT} = \frac{\Delta V_1 + C_1}{V_0 + D_0} \cdot 100$$

which leads to a new definition of WHC

$$WHC_{TOT} = \frac{V_0 - (\Delta V_1 - C_1)}{V_0} \cdot 100$$

This calculation method describes the total change in water holding capacity from raw material to cooked product. As in the traditional way of calculating WHC, the dry matter content of the exudate is included in the calculation.

2.5. Fish material used for validation of the method

2.5.1. Experiments 1 and 2

The test material in these experiments was 12 farmed Atlantic cod (*Gadus morhua*) obtained from Grieg Seafood (Kuneset, Rogaland, Norway) with total body weight before gutting in the range 1.4–2.5 kg with an average of 1.8 kg. The fish was stored on ice, and filleted 2 h *postmortem* while still in *pre-rigor* state.

Samples were prepared as described above. Half of the whole samples (experiment 2) were minced in a grinder with a diameter 70 mm grinding plate (4.5 mm holes) type Forniture TC12F (Sirman, Veneto, Italy), three runs for 5 s each, and carefully mixed to a homogeneous mass.

2.5.2. Experiment 3

The test material was 10 farmed Atlantic cod (*Gadus morhua*) with total body weight after gutting in the range 2.1–2.9 kg with an average of 2.5 kg. All fish were delivered from Fiskeriforskning, Tromsø, originating from Fjord Marin Helgeland AS at Småholman and Langevalen, Brønnøysund, Norway.

The cod was starved for 13 days at an average temperature of 11 °C before slaughter. The fish were killed by a blow to the head and gutted in a pre-rigor state (<5 h post-slaughter) and subsequently sent over night (on ice) to Norconserv AS, Stavanger, Norway. The fish was stored in ice for four days and filleted *post-rigor*.

Samples were prepared as described above. Half of the whole samples (in experiment 3) were vacuum packed in pouches and frozen by dry ice. They were stored for 4, 8 and 10 weeks at −18 °C. After storage they where thawed in air (in a refrigerator) at 4 °C for 16 h.

2.6. Heat-treatment

The cups with the samples were heated in circulating silicon oil (200/20 CS) from 40 °C to 100 °C with 10 °C intervals for 10 min. After heating for 10 min, the samples were cooled in circulating ice water at 0 °C for minimum 90 s to an end temperature in the range 0–1.8 °C.

2.7. Texture

The texture analyses were performed with a Texture Analyser TAXTplus (Stable Micro Systems Ltd., UK), equipped with a 5 kg load cell. A TPA (Texture Profile Analysis) test was conducted for both fillet and myotome samples but with different probes and settings. The TPA test consists of compressing a small piece of food two times in a way that imitates the action of the jaw and extracting from the resulting force–time curve a number of textural parameters that has been found to correlate well with sensory evaluation (Bourne, 1978). The primary parameters of hardness, cohesiveness, springiness (elasticity), and adhesiveness, and the secondary (or derived) parameters of fracturability (brittleness), chewiness and gumminess can be determined by the TPA (Szczesniak, Brandt, & Friedman, 1963).

The myotome samples were measured using a flat ended cylinder of diameter 1/2 in. of type P/0.5 AOAC for gelatine (Stable Micro Systems Ltd., UK). The trigger force for measuring height of the sample was 10.0 g and the cylinder

was moving at 1.0 mm/s until 30% compression of the sample. All samples were stored on ice prior to analysis. Thus, the temperature of the samples was 0–1 °C.

2.8. Statistical analysis

The design of the experiment was created and the results statistically analysed using Minitab R14.13 (Minitab Inc, USA). Cook loss was analysed by the one-way Anova in Minitab with respect to differences in heat treatment and raw material. WHC was analysed as a factorial design with respect to centrifugation speed, heat treatment and raw material.

3. Results and discussion

The sample cups appeared to be practical in use. Mounting the filter had to be done carefully to avoid applying a pressure on the sample while still maintaining good contact between the thin lids and the sample. A small pressure builds up during mounting of the sample cups, indicating that the cup is hermetically closed during heat processing and centrifugation.

3.1. Heating rate of the sample cup and temperature uniformity

It was calculated by the model that the temperature of the surface of the fish will raise to 1 °C below the temperature in the oil bath within maximum 3 s. At the core of a sample of average size (6 mm thick), the temperature will be within 4 °C from the set point after 90 s of heating, i.e., the situation within 15% of the total heating time.

The oil in the heating bath was exchanged by water and temperature distribution and stability was determined with numbered probes placed as shown in Fig. 3. The temperature stability was very good (±0.2 °C), while the temperature distribution was sub optimal. However, the temperature difference between the probes 3–5 was at all times within 1.0 °C.

Heat transfer from water to sample cup was much more effective than from oil. This is shown in Fig. 4 by measurements of the core in 6 mm thick myotomas of cod in the sample cup. On this basis water was chosen for further experiments. Thermo-physical properties of each subdomain including the oil are shown in Table 1. The heat

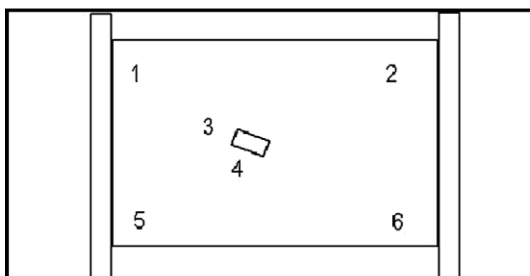

Fig. 3. Sample cup placed in water bath (top view). The numbers indicate positions of each fibre optic temperature sensor.

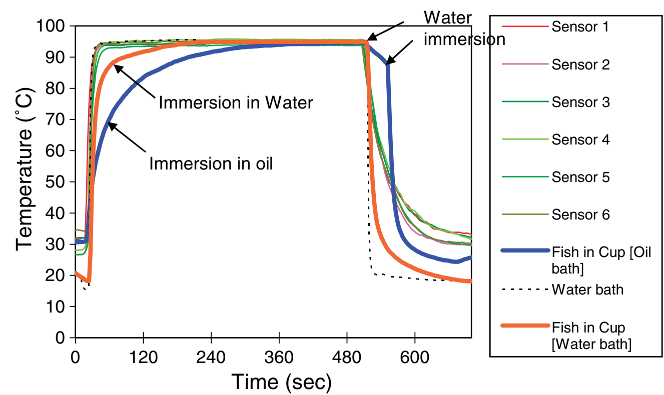

Fig. 4. Temperature measured in fish within sample cup during immersion in water and oil at the same temperature and equal conditions. The sensor numbers are corresponding to position numbers indicated in Fig. 3.

transfer coefficient was calculated by the model to 20,784 W/m^2 for water and 760 W/m^2 for the oil (average for the total surface of the sample cup). The dynamic viscosity of the oil (at 90 °C) was 0.0023 Pa s, which is approximately 10 times higher than for water. This is believed to be the main reason for the much higher rate of heat transfer for water than for oil.

3.2. Ability to measure cook loss

3.2.1. Cook loss in whole and minced fish (experiment 2)

Cook loss of whole and minced samples are shown in Fig. 5 (indicating four samples of each material after heating at 60 °C, 75 °C and 90 °C). A significant difference in cook loss for whole samples between the three temperatures was found. Between 75 °C and 90 °C the results were significantly different ($P < 0.005$) and both were different from the results at 60 °C ($P < 0.002$). As for whole samples a significant difference in cook loss between the three temperatures were found for minced samples. Both at 60 °C and at 75 °C there was a significantly difference from 90 °C ($P < 0.002$) and also in between the results at 60 °C and 75 °C ($P < 0.002$). As shown in Fig. 5, the cook loss is increasing with increasing temperature for both whole and minced samples. Minced samples seem to have a higher cook loss than whole samples, but differences are not statistically significant ($P > 0.05$). Several publications on whole fish reviewed by Aitken and Connell (1979) show cook loss values in the same order of magnitude as found here for both whole and minced samples.

At low temperatures (<40 °C) the main structural changes will appear in the connective tissue due to denaturation of collagen (Lampila & Brown, 1986; Ofstad et al.,

Table 1
Settings for Femlab model of sample cup immersed in circulating oil

Subdomain	Fish	Steel	Oil
Thermal conductivity (W/(m K))	0.5146	44.5	0.14
Density (kg/m^3)	1060	7850	941
Heat capacity (J/(kg K))	3650	475	1500
Initial temperature (K)	275.15	293.15	363.15

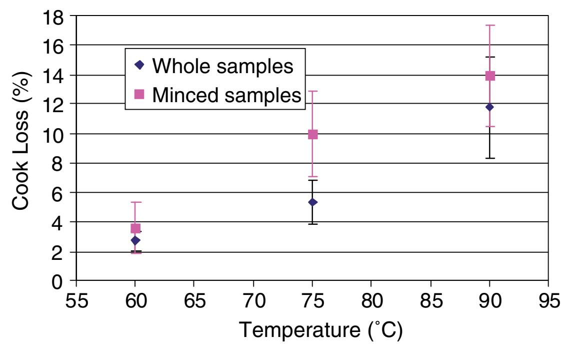

Fig. 5. Mean cook loss of whole and coarsely minced samples at the three different temperatures with standard deviation. Between 75 °C and 90 °C a significant difference was found ($P < 0.005$) and both were different from the results at 60 °C ($P < 0.002$) for whole samples. For minced samples there were also a significant difference between the different temperatures ($P < 0.002$), but the difference between minced and whole samples was not statistically significant ($P > 0.05$).

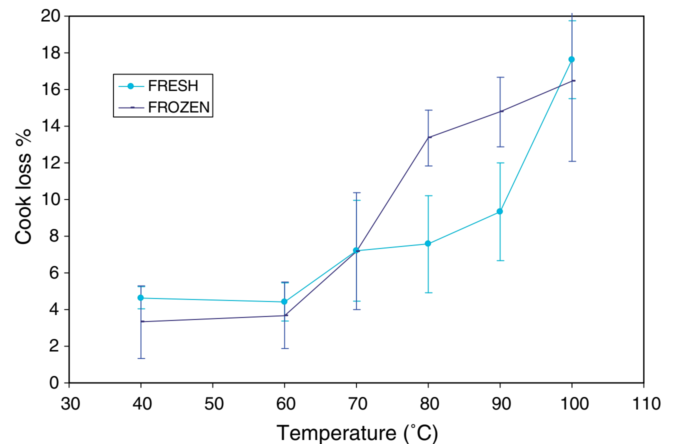

Fig. 6. Cook loss of fresh and frozen whole material after 10 min heating at temperatures from 40 °C to 100 °C ($n = 4$). The difference between fresh and frozen samples is not significant except for 80 °C and 90 °C ($P < 0.05$).

1993; Sikorski, Scott, & Buisson, 1984). Water loss will, however, continue to increase with increasing temperatures due to denaturation of other proteins (Ofstad et al., 1993). It has been shown earlier that maximum decrease in WHC is attained when the muscle cell shrink due to denaturation of myosin and the extra cellular spaces are widened (Ofstad et al., 1993). It is expected to find the highest rate of increase in the cook loss about 45 °C (Ofstad et al., 1993).

3.2.2. The measurement of cook loss as a function of temperature (experiment 3)

Samples were heated for a fixed time of 10 min at different temperatures in the range from 40 °C to 100 °C. Cook loss results are referred in Fig. 6. Both fresh and frozen whole samples were included in the experiment. There is no significant difference between the fresh and frozen samples except at 80 °C and 90 °C ($P < 0.05$). The results for the fresh samples are in the same order of magnitude as for the whole samples in experiment 2. Again the cook loss increases with increasing temperature, but the rather high standard deviations preclude exact conclusions and the variation in results is also larger for the whole samples than for minced. This can be due to the large (biological) variations from fish to fish.

3.3. Ability to measure WHC

Approximately 95% of the water in fish muscle is physically immobilised water, often referred to as "free water". This water is free to migrate throughout the muscle structure and is of interest when measuring the water holding capacity (WHC) of the muscle. Water (or liquid) holding capacity (also called water binding capacity) can be defined as the amount of water left after centrifugation relative to either the water present before centrifugation (WHC_w) or to the dry weight (WHC_d) (Andersen & Jørgensen, 2004). When referred to as liquid holding capacity the amount of expelled fat is also taken into account. The fat content of cod muscle is in the order of magnitude of 0.3%, while the average total liquid loss for non-fatty fish species is 18.6% (Aitken & Connell, 1979). Thus, the nuance between water and liquid holding capacity has been neglected in this paper.

3.3.1. Standard deviation for the method (experiment 1)

The standard deviation of the method was tested with a homogenously ground fish mince. In this test, eight samples were heated at 90 °C for 10 min, cooled and centrifuged together with eight raw samples at 528g. Raw samples gave an average WHC of $82.28 \pm 1.32\%$, while samples cooked at 90 °C for 10 min gave a WHC of $62.22 \pm 2.24\%$. Standard deviation of existing methods has shown to be relative high, for comparison, Ofstad, Kidman, Myklebust, Olsen, and Hermansson (1996) and Sivertsvik and Lorentzen (2004) found the standard deviation to be 10% in their experiments. The standard deviation in this experiment was 1.6% of the average WHC for raw samples and 3.6% for cooked samples. The potential improvement compared to earlier methods can be explained by the fact that the sample is not removed from the cup during processing and analysis. For cooked samples it is assumed that uniform temperature in the sample during processing is one of the most important parameters for maintaining a low standard deviation.

The average cook loss for the eight samples was $7.94 \pm 2.9\%$ of fish weight. The high standard deviation may indicate that the method must be improved before estimating the cook loss of minced samples. It was observed that moist from the air could condense on the outside of the cup and had to be avoided during weighing. Another possible source of error is the dry matter content of the cook loss. Cook loss from samples cooked at 90 °C for 10 min ($n = 20$) was measured to contain $6.05 \pm 0.33\%$ dry matter of total weight of the cook loss.

As the WHC of cooked samples was calculated from the water loss during both cooking and centrifugation, it is possible that the high standard deviation of the cook loss measurements is responsible for the higher standard deviation

in WHC for cooked samples compared to raw samples. Still, a homogenous batch shows to give less variance than samples from individual fish.

3.3.2. Water holding capacity of raw samples (experiment 2)

Just like for earlier methods for measuring WHC by centrifugation, the absolute values found cannot be compared with earlier experiments as the sample cup, centrifugation speed, time of centrifugation, cooking method etc. are different.

WHC of raw samples obtained at different centrifugation speeds are shown in Fig. 7. A significant difference was found between WHC of whole and coarsely minced samples of fish centrifuged at both 326*g* and 528*g*. For whole fish samples there was also a significant difference ($P < 0.05$) between the two centrifugation speeds. This was not the case for minced samples. The reason for this might be that the chopping of the muscle has already caused severe changes in the muscle structure, and increased centrifugation speed will therefore not affect the WHC of the muscle in the same way that it will for whole muscle.

The properties of finely comminuted cod is affected both by the structure of the biological tissue and the new structures formed during processing (Ofstad et al., 1996).

As expected, the WHC of coarsely minced samples was lower than the WHC for whole muscle. Compared to the findings in experiment 1, rather large standard deviations are observed, especially for the minced samples. For both whole and minced fish, material from three different fishes was used, which could explain this variation.

It is generally accepted that the forces that immobilise "free water" within the muscle are generated by surface tension (Hamm, 1986). More specifically, the water is held trapped within the muscle by capillary forces (Trout, 1988). The capillary forces are caused by the pores located between myosin and actin, which under normal conditions are approximately 10 nm (Hermansson, 1983).

3.3.3. WHC fresh and frozen whole samples as a function of temperature (experiment 3)

Fig. 8 shows the results from measurement of WHC after heat treatment at temperatures in the range 40–100 °C, including raw samples. Again, the WHC is shown to decrease with increasing temperature. Compared to the results for experiments 1 and 2 the results are in the same range.

The difference between fresh and frozen raw material is not significant. This is in agreement with Schubring (2005) who found no significant changes in texture and WHC for raw cod stored for 13 months at −28 °C. During heating for 10 min at 40 °C before centrifugation there is, however, a much lower WHC for frozen samples. However, this difference could not be seen at higher temperatures.

For samples of frozen fish the WHC is decreasing with increasing temperature, which is expected. For fresh samples there seems to be an increase in WHC at 80 °C compared to 60 °C and 70 °C, but this is not a statistically significant increase.

The heating time was 10 min in all the experiments presented here and leaves the question of the effect of a variable heating time open. However, based on Ofstad et al. (1996) it can be expected to find decreasing WHC with increasing holding time. This may be a good basis for elaborating experiments to obtain kinetic parameters. As discussed in the introduction, water loss and decrease in WHC is expected to be due to denaturation of several proteins. Experiments on protein determination using differential scanning calorimetry has shown from three to five protein denaturation peaks (Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002).

3.4. Ability to measure texture

The average maximum force for 30% compression of myotomes in the sample cup is shown in Table 2. The standard deviation of the raw samples was 16% of the total maximum average force and the difference between the

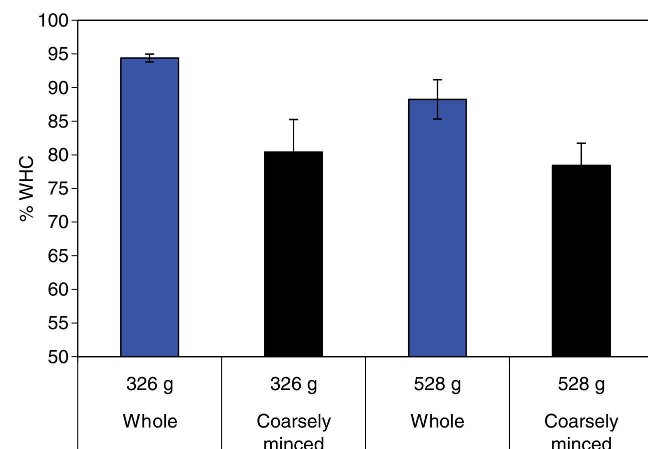

Fig. 7. Raw samples (whole and coarsely minced) at two different rotation speeds with standard deviation. The results for whole and minced samples centrifuged at 326*g* and 528*g* was significantly different ($P < 0.05$).

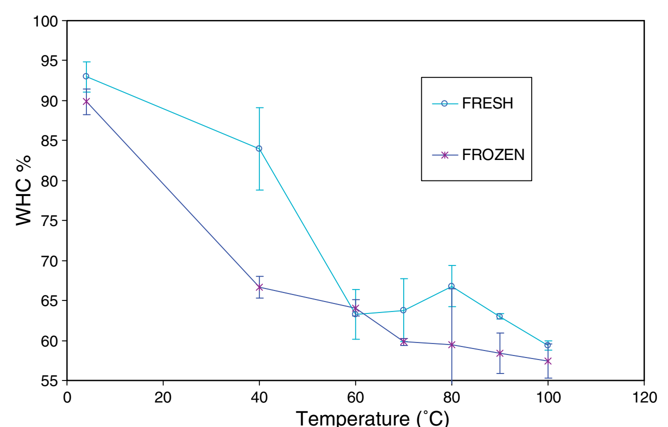

Fig. 8. WHC measured with the sample cup after 10 min heat treatment at different temperatures with both frozen and fresh whole, raw material ($n = 4$). The difference between fresh and frozen raw material is not significant ($P > 0.05$).

Table 2
Average maximum force of cooked material in g for a TPA study on myotoma in sample cup at 30% compression ($n = 6$)

Treatment	Hardness, maximum average force (g)	Standard deviation	Standard deviation in % of hardness
Raw	139	23	16
10 min at 75 °C	468	67	14
10 min at 90 °C	776	133	17

A significant difference was found between raw and the two heat treated groups of samples ($P < 0.0001$).

raw and the two heat treated groups of samples was statistically significant ($P < 0.0001$). In spite of the high standard deviations, it seems feasible to use this method for characterising differences in texture of raw and heat treated myotoma samples.

4. Conclusion

It is feasible to use the new method and associated equipment (sample cups) for rapid and uniform heating of samples and after heating and cooling gravimetrically determine the cook loss with a standard deviation within 30% of the total cook loss for minced samples. Both intact and coarsely minced muscle of farmed cod was used to investigate the abilities and limitations of the method. Determination of WHC for whole, raw and cooked samples gave expected results. There were, however, significant differences between the results for similar samples measured under similar conditions.

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Paper II

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Kinetics of heat denaturation of proteins from farmed Atlantic cod (*Gadus morhua*)

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Abstract

Protein denaturation is considered to be the main reason for both water loss and textural changes in fish during thermal processing. Denaturation of proteins in muscle of farmed Atlantic cod was studied by differential scanning calorimetry. The denaturation of the proteins was compared to cook loss and loss in water holding capacity and it was shown that the protein denaturation occurs in a lower temperature range (35–66 °C) than the appearance of major cook loss (above 80 °C) when cod muscle is heated. Other mechanisms for release of water than protein denaturation should therefore be investigated.

Kinetic parameters for changes in denaturation enthalpy of cod protein were estimated in the temperature range of 58–68 °C, corresponding to the denaturation of actin. The time-dependent decrease in denaturation enthalpy corresponded to a first-order mechanism. The decimal reduction time $D_{62\,°C}$ was estimated to be 130.1 ± 5.4 min and the *z*-value 5.74 ± 0.11 °C.
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Keywords: Thermal processing; Kinetic; Protein denaturation; Cod; *Gadus morhua*; Farmed fish

1. Introduction

Minimally processed convenience foods are a growing segment in the European marketplace. Fish based products are underrepresented among these foods, due to a number of unsolved problems. To the consumer the most obvious quality parameters related to fish are flaking (whether the fish is falling apart on the plate or not), tenderness and juiciness. For a species like cod these challenges are related to seasonal variations and combined with the decreasing stock of wild cod and the increasing market demand, farming of cod has become interesting. Shelf life and microbial safety are other important parameters. For convenience foods it is commonly accepted that mild thermal processing of fish products should at least provide a 6 log inactivation of *Listeria monocytogenes*. Ben Embarek (1993) investigated the heat resistance of *L. monocytogenes* O57 in cod and found a decimal reduction time $D_{70\,°C}$ of 0.05 min and a *z*-value of 6.2 °C. Accordingly, a 6 log inactivation would require a pasteurisation value $P_{70\,°C}$ of minimum 0.30 min. More general literature suggests a minimal pasteurisation value $P_{70\,°C}$ of 2 min (European Commission, 1999). To achieve this heat load at the cold spot of the product, holding times and/or temperatures that may have undesirable consequences with regard to quality might have to be used. Protein denaturation has earlier been considered to be the main reason for both water loss and textural changes in fish during thermal processing (Ofstad, Kidman, & Hermansson, 1996). Therefore protein denaturation studies can reveal the mechanisms behind these major quality changes. Knowledge of kinetic parameters for thermal protein denaturation can also be used for design and optimization purposes. One of the methods used for characterisation of thermal protein denaturation in fish is differential scanning calorimetry (DSC) (Hastings, Rodger,

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Park, Matthews, & Anderson, 1985). None of the aforementioned publications presented kinetic data for heat-induced denaturation of proteins from cod.

Therefore, the purpose of this study was to quantify the kinetics of protein denaturation during thermal processing of farmed Atlantic cod using DSC. Furthermore, the protein denaturation was to be compared to cook loss and loss of water holding capacity (WHC) and the possibility of using this information for optimization of mild thermal processing of vacuum packed convenience fish products was to be discussed.

2. Materials and methods

2.1. Raw material

Farmed atlantic cod (*Gadus morhua*) (94 individuals) were obtained from an aquaculture station (Havbruksstasjonen AS, Skulgambukt, Tromsø, Norway). The fish were hatched autumn 2003 and fed, starting from February 2005, with a commercially available feed (BioMarine Basic, Biomar AS, Myre, Norway) with a protein content of 50% and fat content of 16%. The fish were starved for one week at an average temperature of 7.0 °C before slaughtering. The fish were slaughtered by a blow to the head, cut by the throat and bled for 30 min in a container of seawater at 6.9 °C. The fish were kept on ice during transportation and gutted the next day while still in the state of *rigor mortis*. The average weight of the round, whole fish was 3651 ± 587 g. The fish were sorted by gender (57 male and 37 female). Average weight before gutting was the same for either gender, while the average milt weight ($n = 10$) was 469 ± 166 g and the average roe weight ($n = 10$) was 232 ± 49 g. Average length was 646 ± 38 mm for male fish and 650 ± 30 mm for female. Average liver index ($n = 20$) was 0.101 ± 0.018 g liver per g fish.

After an additional 2 days of storage at 2 °C (post *rigor mortis*), the fish were filleted, deep skinned (removing connective tissue) and cut to loins. 55 loins of each gender (a total of 110) were coarsely ground in a grinder with Ø8 mm holes (T. Myhrvold AS, Oslo, Norway) and mixed manually. The mixture was packed in polyethylene beakers (Dynopack no. 567, Polimoon AS, Kristiansand, Norway) of 125 g each, spread over 22 beakers of single gender and 182 beakers of mixed raw material (50% of each gender) and sealed under vacuum by a PA/PE-film (15/75 my respectively) before freezing. The beakers were stacked between layers of dry ice and stored in a freezer at -80 ± 1 °C until further use. The rest of the fresh fish material was used for analysis of raw material parameters.

2.2. Characterisation of raw fish

The pH was measured using a pH meter (Orion 410Aplus Benchtop, Thermo Electron Corporation, MA, US) equipped with a puncture combination electrode (81-63 ROSS™, Thermo Electron Corporation, MA, US). Total protein content was analysed based on the method of Kjeldahl (1965). The water and ash content was determined gravimetrically following drying for 16–18 h at 105 °C and 550 °C, respectively. Total volatile Nitrogen (TVN) and trimethylaminoxide (TMAO) was determined using a modified Conway microdiffusion method (Conway & Byrne, 1933). The results are expressed as mg N/100 g of raw material.

For microbial analysis, samples of 25 g ground cod were homogenized in 250 ml of 0.9% NaCl (w/v) and 0.1% peptone (w/v) for 120 s in a Stomacher 400 Laboratory Blender (Seward Medical, London, UK). Aerobic plate counts (APC) were determined in aliquots from suitable dilutions added to melted and tempered (44 °C) iron agar with overlay (Agar Lyngby, IA, Oxoid CM 867, Basingstoke, Hampshire, UK) supplemented with L-cysteine, and incubated at 20 ± 1 °C for 3 days. Black colonies were counted as H_2S-producing bacteria, APC were counted as the total of black and white colonies. The content of psychrotrophic bacteria was determined by a spread plate count method with plate count agar (PCA, Merck, Darmstadt, Germany) with 1% NaCl added, in order to support growth of salt requiring *Photobacterium phosphoreum*, and incubated at 8 °C for 5–7 days. Average results of duplicate measurements are presented as log colony-forming units per gram muscle (log cfu/g).

2.3. Sample preparation

After 1 month of storage at −80 °C, the material was used over a period of 3 months. The frozen material form each beaker was divided by a chisel and crushed and mixed frozen in a grinder (Retsch Grindomix GM200, Retsch GmbH, Haan, Germany) to a fine, homogenous powder. The fish powder was thawed on ice and kept at 0 °C until filling of 69 ± 1 mg in stainless steel DSC-pans Ø72 mm and height 24 mm (Perkin–Elmer, Norwalk, USA) with a spatula. Each pan was sealed with a rubber ring and vacuum packed in a plastic bag (low density polyethylene) of approximately 30×50 mm.

2.4. Heat treatment

Samples were heat treated in a water bath at constant temperature (Julabo 5B, Julabo Laboratorietechnik GmbH, Seelbach, Germany) except for 2 untreated reference samples in each set (a total of 13 untreated samples). In a first set of experiments, the stainless steel DSC-pans were heated for 10 min at constant temperature in the range of 26–80 °C. This was performed as a screening study which allowed concluding that preheating for 10 min at 52 °C would leave only one peak on the thermogram. After this preheating, the samples were heated for preset times (2–280 min) at constant temperatures in the range of 56–68 °C. As a reference, for each set of heat treatments, 3 pans were only pre-treated.

All samples were cooled in ice water immediately after heat treatment and stored at 0 °C overnight until recording of DSC-thermograms. In this way only irreversible changes in denaturation enthalpy were considered.

Temperature abuse was carefully avoided by keeping the samples in an isolated box. The samples were kept at a temperature around 0 °C between each step of the experiments. However, filling and weighing was done within 3 min in ambient room temperature ranging from 19 °C to 20 °C. During this period the samples may have reached room temperature due to the fact that both spatulas and pan have large surface areas and a high heat capacity compared to the fish. After sealing of the pan the sample was still vulnerable to temperature abuse as the pan was designed for rapid heating and cooling. Body heat was a possible discrepancy as the temperature of the water bath was only 26 °C in part of the isothermal screening study. However, during the kinetic experiment, the influence of a possible temperature abuse during sample preparation was minor compared to the pre-treatment of 10 min at 52 °C.

2.5. DSC analysis

DSC was performed at a heating rate of 10 °C/min over the range from 0 °C to 110 °C on a Perkin–Elmer DSC7 (Perkin–Elmer, Norwalk, USA). Empty pans were used as reference and 2 min equilibration at 0 °C was done before each run. The residual denaturation enthalpy (ΔH), was defined as the area under the denaturation peak using a straight base line as shown in Fig. 1. The calculation of area was made by Pyris software (Perkin–Elmer, 1996). Samples were measured in triplicate. For determining the start point of the area under peak 5 (at ~30 °C), the derivative curve was used to determine where the slope of the heat flow curve changed. The end point was defined by the local minimum of the heat flow curve where the derivative curve again reaches a constant value as shown in Fig. 1.

2.6. Kinetic data analysis

Heat denaturation of proteins can often be described by a first-order reaction (Eq. (1)).

$$\frac{dX}{dt} = -kX \tag{1}$$

where X is a response value, in this case the residual denaturation enthalpy (ΔH), and k the reaction rate constant. In case of isothermal experiments (i.e. rate constant k not varying with time), Eq. (1) can be integrated, yielding Eq. (2):

$$X = X_0 \exp(-kt) \tag{2}$$

In the area of food science and technology, it is common to characterise first-order reactions using the Thermal Death Time concept. The decimal reduction time (D-value) is the time, at a given constant temperature, needed for a 90% reduction of the response value. For a first-order reaction, D-values and rate constants are inversely related (Eq. (3)).

$$D = \frac{\ln(10)}{k} \tag{3}$$

Substitution of Eq. (3) into Eq. (2) yields an alternative equation for a first-order reaction (Eq. (4)).

$$X = X_0 10^{-\frac{t}{D}} \tag{4}$$

The validity of a first-order denaturation can be examined by plotting residual response value versus treatment time

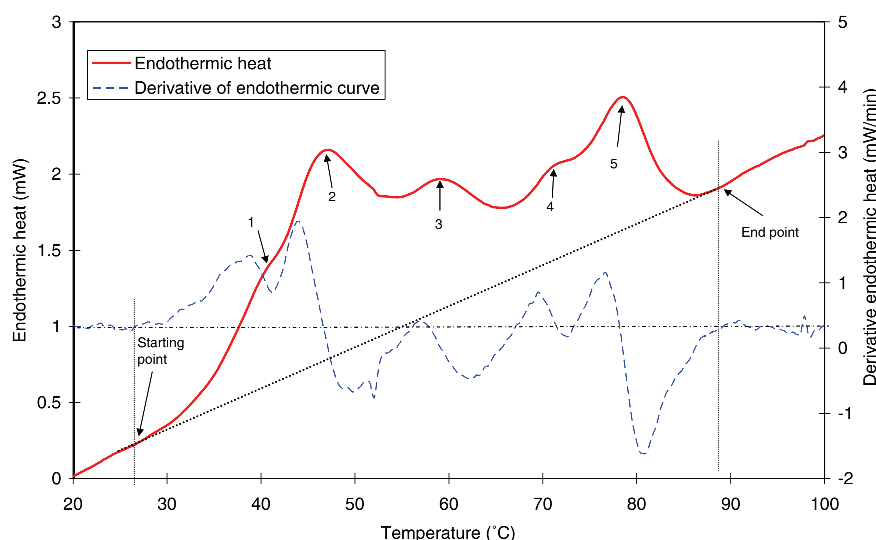

Fig. 1. Typical DSC-thermogram (continuous line, heating rate of 10 °C/min) of untreated cod muscle. Numbers indicate the position of the five peaks listed in Table 1. The dashed line corresponds to the derivative of the heat flow curve and is used to establish the positions of the peaks. The total peak area is defined as the area between the red curve and the baseline (dotted line). Starting point of the area was found by the inflection point of the heating curve. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

on a semi-logarithmic scale and evaluation of the goodness-of-fit.

The temperature dependence of the rate constant k is often expressed by an activation energy, E_a, as indicated in the Arrhenius relationship (Eq. (5)):

$$k = k_{ref} \exp\left(\frac{E_a}{R_g}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right) \quad (5)$$

where k_{ref} is the inactivation rate constant at T_{ref}, T_{ref} the reference temperature, E_a the activation energy and R_g the universal gas constant ($R_g = 8.314$ J/K mol).

In the Thermal Death Time model, the temperature dependence of the D-value is given by the z-value (Eq. (6)). The z-value equals the temperature increase necessary to obtain a tenfold decrease of the D-value.

$$D = D_{ref} 10^{\frac{T_{ref}-T}{z}} \quad (6)$$

where D_{ref} is the decimal reduction time at T_{ref}, T_{ref} the reference temperature, and z the z-value.

By combination of Eq. (4) with Eqs. (6), (7) is obtained from which the kinetic parameters for heat denaturation of actin, i.e. D_{ref} and z, were estimated by a nonlinear regression analysis (SAS Institute Inc., 2003).

$$X = X_0 10^{-\left(\frac{t}{D_{ref} 10^{\frac{T_{ref}-T}{z}}}\right)} \quad (7)$$

2.7. Cook loss and WHC analysis

Cook loss and WHC was obtained by the method and associated equipment described in our previous study (Skipnes, Ostby, & Hendrickx, 2007). The samples ($n = 8$) were placed in a sample cup which was sealed before isothermal heating in a water bath for 10 min at temperatures in the range 25–95 °C. After heating the samples were cooled and centrifuged together with 8 raw samples at 528g for 15 min.

Cook loss was defined as the weight of exudates during cooking in percent of initial fish weight and WHC was determined as the total weight of cook loss and liquid expelled during centrifugation in percent of the initial water content of the fish. Dry matter content of fish muscle was determined gravimetrically after drying at 105 °C for 16 h.

3. Results

3.1. Characterisation of raw fish

Ground fish material pH was measured ($n = 2$) to 6.22 ± 0.01 for the mixture, while 6.27 for male and 6.14 ± 0.04 for female respectively. Average water content ($n = 8$) was $78.65 \pm 11\%$ (w/w), fat ($n = 3$) 0.56%, protein ($n = 3$) 20.1%, ash ($n = 3$) 1.18% and salt ($n = 3$) 0.105%. Average content of TMAO ($n = 2$) for the mixture consisting of both gender was 32.2 ± 1.3 mg N/100 g fish, while 38.6 ± 4.5 mg N/100 g for female and 16.0 ± 1.2 mg N/100 g for male. Average total volatile N for all three mixtures ($n = 2$) was 13.25 ± 0.66 mg N/100 g fish mixture. Total CFU for the mixture was 35,000/g of which 3200/g psychrotropic bacteria. No H_2S-producing bacteria were detected. Average thermal conductivity ($n = 28$) was 0.42 ± 0.03 W/m K. The content of TMAO and total CFU indicates that the first stage of the degradation process had started, but the values are not much higher than for fresh fish and none of the other values indicate any degradation.

3.2. DSC-thermograms from untreated samples

The denaturation enthalpy ΔH is a net value of endothermic and exothermic reactions and is correlated with the remaining content of ordered three-dimensional structure of a protein (Ma & Harwalkar, 1991; Privalov & Khechinashvili, 1974). Thus, a reduction in residual enthalpy is an indication for a partial loss of protein structure during the preceding heat treatment. Below, the residual denaturation enthalpy of untreated cod muscle is presented.

Five denaturation peaks were found in the untreated cod muscle as shown in Fig. 1. Total denaturation enthalpy determined as the total area of all peaks, i.e. the heat absorbed during the DSC, was 3.242 ± 0.196 J/g ($n = 13$). The number of denaturation peaks described in the literature for cod proteins range between 3 and 8 when scanning from 0 °C to 110 °C (Hastings et al., 1985; Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002). Studies of isolated cod proteins by DSC have shown that these peaks correspond to denaturation of specific proteins (Hastings et al., 1985). Several of the peaks found are composites of the transition of two proteins; e.g. the first peak found for cod corresponds to the denaturation of collagen and sub-fragments of myosin. As the purification process of the proteins may reduce their heat stability and alter the peak maximum temperature (Thorarinsdottir et al., 2002), the correspondence between the peaks of the individual, isolated proteins and the individual proteins in the mixture is not always obvious. Sub-fragments of myosin from rabbit muscle have also been studied by DSC and the denaturation peaks associated with thermal unfolding of the helical tail, the hinge-region and the globular heads of the myosin molecule corresponded to the peaks in thermograms for myosin (Wright & Wilding, 1984).

Hastings et al. (1985) isolated connective tissue from the skin of cod as a source of collagen and two denaturation peaks were found at ~32 °C and ~40 °C respectively. Myosin and sarcoplasmic proteins were extracted from cod meat and showed 4 peaks each, while actin was isolated from an acetone powder and showed a peak at ~81 °C. When analyzing the cod meat, a total of 8 peaks for the cod meat corresponding to peaks for the isolated proteins were found. The five peaks detected in the present study are listed in Table 1 (together with literature data); represent denaturation of myosin (peaks 1 and 2), sarcoplasmic

Table 1
Average values and standard deviation for peak maximum temperature (T_{max}) for untreated cod based on the current work and literature data

Material	Apparatus/heating rate	Peak 1 (°C)	Peak 2[a] (°C)	Peak 3[b] (°C)	Peak 4 (°C)	Peak 5[c] (°C)	References
Farmed frozen (−80 °C)	DSC-7/10 °C/min	38.4 ± 0.7	44.1 ± 0.2	57.3 ± 0.1	69.5 ± 0.3	76.1 ± 0.7	Present work ($n = 13$)
Wild, natural, frozen (−30 °C)	DSC-7/10 °C/min		~45	~54		70–80	Jensen and Jorgensen (2003)
Wild, natural, frozen (−24 °C)	DSC-7/10 °C/min		43.5 ± 0.2	59.3 ± 0.9		73.6 ± 0.7	Thorarinsdottir et al. (2002)
Wild and fed, fresh	Setaram micro DSC/ 1 °C/min		~42	~56–57		~73–74	Ofstad et al. (1996)
Wild, fresh	DSC-2/10 °C/min		~44	~58		~76	Hastings et al. (1985) (1 fish)
Wild, fresh	DSC-25 °C/min		~44	~54		~74	Poulter, Ledward, Godber, Hall and Rowlands (1985) ($n = 2$)
Wild, frozen	DSC-25 °C/min		~42	~52		~74	Poulter et al. (1985) ($n = 2$)

[a] Myosin.
[b] Sarcoplasmic proteins.
[c] Actin.

proteins (peaks 3 and 4) and actin (peak 5). The amount of collagen in the muscle is probably too low to be detected as a peak, or may be covered by the first myosin peak (peak 1) as it is supposed to be denatured at temperatures as low as 30–40 °C. There was no indication of any additional peaks covered under the five peaks discussed. Compared to the reported values in the literature, the peak maximum temperatures (T_{max}) have been established with a high degree of accuracy. This might be due to the fact that the experiments are based on a large number of cod ground and mixed into one homogenous batch. In this way the influence of the possibly large differences between each individual fish is reduced to a minimum. The denaturation temperatures obtained for farmed cod in this experiment are in good agreement with the values found for wild cod in literature even though some of the authors used other heating rates.

Rescanning of DSC pans in the range 0–65 °C or in the range 0–110 °C confirmed that the denaturation was irreversible as no peaks were detected during the second scan.

3.3. Isothermal screening of protein denaturation

Protein denaturation was screened by heating for 10 min at temperatures in the range of 26–80 °C and determining the residual ΔH.

In Fig. 2, some typical thermograms obtained by differential scanning calorimetry of heat-treated cod muscle are shown. When scanning the samples previously heated in a water bath for 10 min, the peak heights of the endothermic curve decreased with increasing temperature. The values for T_{max} and areas of the curves from samples heated at 26–34 °C were the same as for untreated samples. Peaks 1 and 2 were strongly reduced after heating at 36 °C for 10 min, and at higher temperatures they disappeared.

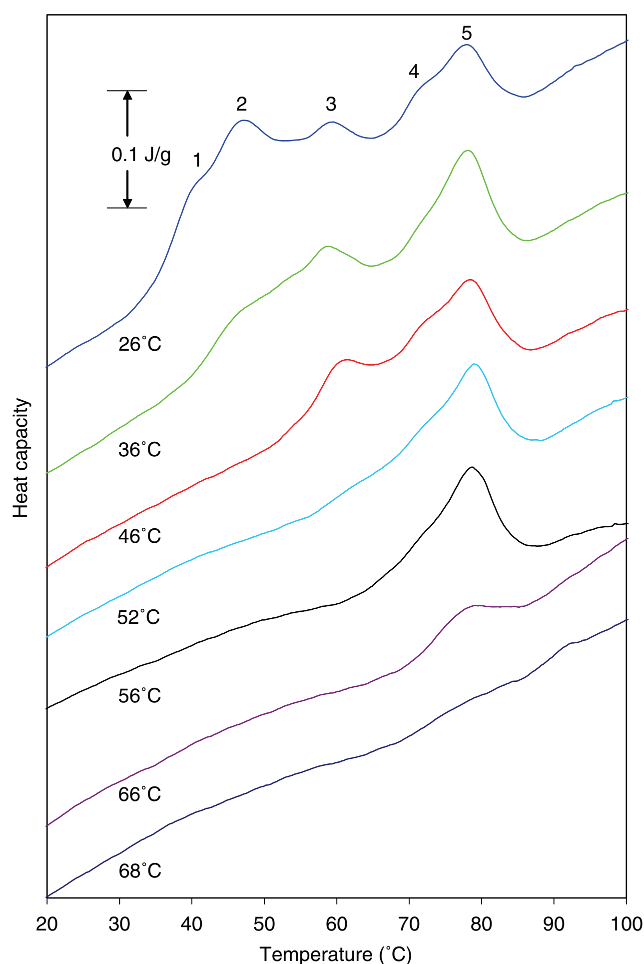

Fig. 2. Typical DSC-thermograms (heating rate of 10 °C/min) of cod muscle heated for 10 min at 26 °C (peaks similar to untreated), 36 °C, 46 °C, 52 °C, 56 °C, 66 °C and 68 °C (from top to bottom).

With increasing temperatures up to 68 °C, the 5 peaks diminished gradually and according to the denaturation

temperature, until no peak could be detected (Fig. 2). However, further unfolding of proteins cannot be precluded if additional heat load is applied.

The residual denaturation enthalpy was calculated relative to the average denaturation enthalpy of the preheated samples. As shown in Fig. 3, a gradual decrease in residual enthalpy occurred when heating at higher temperature, corresponding to unfolding of the cod muscle proteins. Temperature thresholds for changes in ΔH are lower than the onset temperatures of the peaks observed in the thermogram, as a result of the difference in time scale (10 min in comparison to a few seconds). At temperatures above 66 °C, the peak area was either zero or too small to be measured. In the same figure, also cook loss and water holding capacity (WHC) of cod isothermally heat-treated for 10 min are shown. Skipnes et al. (2007) showed that the cook loss was moderate during heating in the range 40 °C to 60 °C and increased rapidly from a temperature between 60 °C and 70 °C and up to 100 °C. From Fig. 3 a similar trend can be seen, but the major increase in cook loss is shown to be at a temperature above 80 °C, i.e. in a temperature range where ΔH is 0 and changes in WHC are small. For optimization of mild heat treatment this is of interest as it opens up the possibility of a process with denatured proteins and thus a product with cooked appearance, while the cook loss is still moderate. In the same temperature region it will also be possible to adequately inactivate *L. monocytogenes*. The shelf life will however be limited as long as other pathogens, i.e. non-proteolytic *Clostridium botulinum* type E, and their spores are not inactivated.

As shown in Fig. 3, WHC is decreasing sharply with increasing temperature from 30 °C and to 40 °C which is coincident with the first drop in ΔH. However, from the previous data (Skipnes et al., 2007) it could be expected a stable or decreasing WHC with increasing temperatures above 55 °C, while the data in Fig. 3 indicates an increase in WHC from 55 °C compared to the WHC at 50 °C ($p = 0.96$). Slow heating (1 °C/min) of coarsely chopped cod to a specified temperature showed higher liquid loss at 45 °C compared to temperatures from 50 °C to 70 °C (Ofstad, Kidman, Myklebust, & Hermansson, 1993), which is similar to the behaviour of the WHC curve shown in Fig. 3. Based on micrographs of cross sections of the muscle Ofstad et al. (1993) suggested that aggregates of sarcoplasmic proteins and collagen are able to hold water and/or plug intercellular capillaries and prevent water release during centrifugation. The changes in WHC with temperature may also be a result of a change in the proportion of water released form the sarcoplasm and myofibrils as this also may be a function of temperature. WHC has been used as indicator for protein denaturation and as a measure for quality of raw fish (Rustad, 1992) where the protein denaturation is part of the non-thermal degradation of fish meat. There is also a relation between heat denaturation of proteins and WHC. The decrease in WHC continues at temperatures above 66 °C, when ΔH is decreased to nearly 0. Thus, the reduction in WHC cannot be explained by the reduction in ΔH and WHC may be used only partly for characterizing protein denaturation during thermal processing.

3.4. Kinetics of cod protein denaturation

For the kinetic study on the heat induced unfolding of cod proteins as measured by DSC, only the unfolding of actin (peak 5 in Fig. 1) was considered. After preheating at 52 °C for 10 min, the average enthalpy of the only remaining peak on the thermograms was 0.954 ± 0.070 J/g.

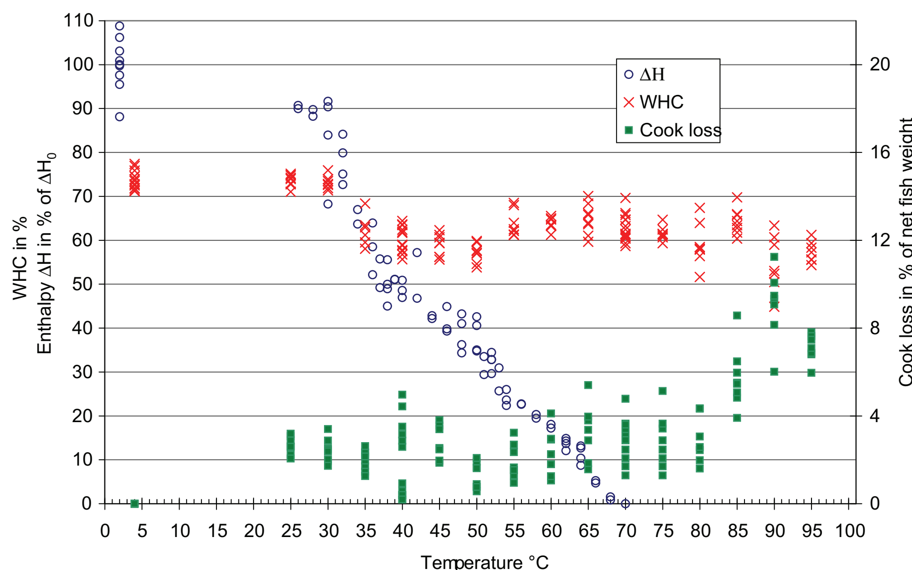

Fig. 3. Residual denaturation enthalpy ΔH of all peaks (in % of ΔH of the untreated cod muscle, ○), water holding capacity (in % of initial water content, ×) and cook loss (in % of net fish weight, ■) of cod muscle after 10 min heat treatment.

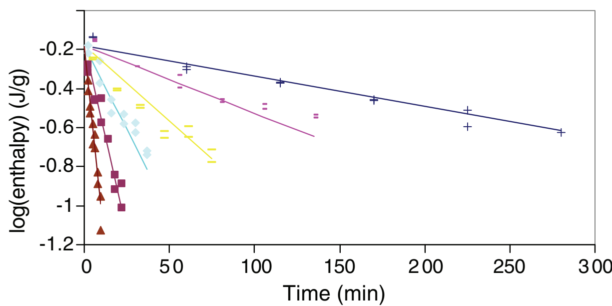

Fig. 4. Logarithmic plot of the residual denaturation enthalpy of cod muscle after isothermal treatment at 58 (+), 60 (-), 62 (–), 64 (♦), 66 (■), and 68 °C (▲) as a function of treatment time (min). The cod muscle was preheated to 52 °C for 10 min to obtain data for actin only. The full lines represent the model fitting (Eq. (1)).

Table 2
Kinetic parameters for heat denaturation of actin in cod muscle

Parameter	Estimate	Standard error	Approximate 95% confidence limits	
D_{ref} (at 62 °C) min	130.1	5.4	119.3	140.8
z (°C)	5.74	0.11	5.52	5.97
H_0 (J/g)	0.658	0.014	0.6313	0.6855

According to earlier findings this peak is expected to mainly be the result of actin denaturation (Hastings et al., 1985). Compared to the findings for isothermally heating to temperatures of 38 °C for 10 min, both the residual enthalpy of the peak and the standard deviation are lower. Increased accuracy was expected as the samples were preheated to 52 °C.

In Fig. 4, the logarithm of the residual denaturation enthalpy is plotted against holding time for each of the temperatures used for heat treatment in this experiment. The data were used for the global regression model and regression lines are shown together with the measured data. The denaturation enthalpy of cod muscle at zero treatment time (i.e. only pre-treatment), ΔH_0, is a total of remaining myosin, sarcoplasmic proteins and actin and was ignored in the regression model as the remaining myosin and sarcoplasmic proteins would interfere with parameter estimation. However, after the second heat treatment from 56 °C and upwards, residual myosin and sarcoplasmic proteins is not expected to be of significance as it could not be detected after heat treatment at these temperatures in the screening study.

When the ΔH_0 values was excluded, the regression lines showed a good fit for a first-order denaturation reaction for all of the temperatures measured. From these observations, kinetic data for the protein denaturation could be estimated for the actin peak and the results are presented in Table 2.

The $D_{62\,°C}$ for actin was estimated to be 130.1 min (Table 2). For comparison, the D-value of *L. monocytogenes* O57 at 62 °C is 0.98 min (calculated from Ben Embarek (1993)) while the recommendations from ECCF (1996) are based on a $D_{62\,°C}$ of 3.85 min. Thus, actin is more resistant to heat denaturation than *L. monocytogenes.*

Another possible use of these results could be to determine based on the temperature history whether the fish will appear to be cooked or not. Proteins not denatured will appear raw to the consumer, which is undesirable for most fish meals. A correlation between results of sensory analysis and cod protein denaturation should therefore be investigated. An optimized mild thermal process should not only inactivate *L. monocytogenes* but also be sufficient to denature proteins to give a cooked appearance. The determined kinetic values should therefore be used to calculate the adequate heat load.

4. Conclusion

By means of DSC the thermograms for farmed Atlantic cod (*G. morhua*) have been recorded and the enthalpy of protein denaturation obtained in the temperature range 0–110 °C. Kinetic parameters for first-order protein denaturation in the temperature range 58–68 °C, corresponding to denaturation of actin, were determined to a $D_{62\,°C}$ of (130.1 ± 5.4) min and a z-value of (5.74 ± 0.11) °C. In order to optimise the thermal processing of vacuum packed convenience fish products it is of interest to avoid cook loss while obtaining a cooked appearance and microbial safety. The results in this study indicate that the required heat load to denaturate actin is higher than for *L. monocytogenes*, while the cook loss is moderate at the same heat load. The kinetic data obtained may therefore be used for optimization purposes.

When heating cod, the proteins are denatured before the major cook loss occurs and the fish will appear to be cooked without a major cook loss. However, the water holding capacity is significantly reduced already around 35 °C, i.e. as soon as the temperatures are high enough to denature collagen and myosin. Kinetic values for cook loss and WHC should be established in order to combine values for microbial inactivation with quality related parameters. Since the reduction in WHC and the increase in cook loss during heating of cod can only partly be correlated to protein denaturation, other mechanisms for release of water (e.g. thermodynamic changes in capillary forces) should be investigated.

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Paper III

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ORIGINAL PAPER

Thermal Inactivation kinetics of acid phosphatase (ACP) in cod (*Gadus morhua*)

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Abstract Thermal inactivation of acid phosphatase (ACP) in muscle extracts of farmed cod was measured in the range of 55–67.5 °C obtaining $D_{60} = 6.78 \pm 0.10$ min and $z = 6.37 \pm 0.09$ °C. These data show that ACP is less thermostable than heat-resistant pathogen micro-organisms (e.g. *Listeria monocytogenes*) and therefore could be inactivated in heat-preserved fish products. As a consequence, residual ACP activity is not recommended as a quantitative intrinsic time temperature indicator (TTI) relative to pathogenic micro-organisms, based on these preliminary studies. Hence, ACP residual activity is probably not a shelf life limiting factor of cod products but might have its potential as an indirect monitor of quality parameters during thermal processing.

Keywords Acid phosphatase · Thermal inactivation · Kinetics · *z*-value · Cod · Fish

Introduction

Thermal processing is one of the most common methods to achieve safe convenience foods with extended shelf life. In thermal processing, the aim is often to apply the minimum thermal load required to destroy pathogenic and spoilage micro-organisms and enzymes [1, 2]. “Sous vide” products are an alternative to fresh products, where mild thermal processing of vacuum-packed products is used in order to preserve the taste and texture. Raw material quality, processing conditions and methods, potential micro-organisms to be controlled, as well as storage conditions and required shelf life must be known in order to design a thermal process for a specific product.

The quality of the fish is severely reduced if the thermal process is designed for a shelf life of more than 21 days at chilled conditions, requiring at least a 6 log inactivation of non-proteolytic *Clostridium botulinum* [3]. Designing a thermal process for such a product is challenging as the heat load required for inactivating micro-organisms and enzymes may cause undesirable changes such as a dry structure and flaking. Reduced cooking times at lower temperature is preferred with respect to the sensory quality of fish products, but this will reduce the shelf life [4, 5]. For processing in the temperature range of 60–75 °C, several pathogenic vegetative micro-organisms could pose a threat to product safety. Inactivation of *Listeria monocytogenes* has been suggested as a criterion for minimum heat treatment by the European Chilled Food Federation (ECFF) [6]. A number of enzymes are also inactivated in the same temperature region. Knowledge of the inactivation kinetics of enzymes can be used to determine its potential as intrinsic time temperature indicators (TTI) for determining either microbial inactivation or the change in a specific quality parameter. A TTI is defined as a small measuring device that shows a time–temperature dependent, exact irreversible change that mimics the change of a target attribute exposed to the same variable temperature exposure [7].

The knowledge of their inactivation is of interest for two reasons. First, some of them play a role in the breakdown of the product and limit the shelf life. Secondly, they could be of interest as TTI mimicking inactivation of pathogenic micro-organisms. Enzymes could also be of interest as a

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TTI for indirect monitoring of quality parameters during thermal processing, especially for quality parameters that are difficult to measure like degree of protein denaturation. Sarcoplasmic proteins of cod are denatured around 54 °C and myosin around 45 °C [8]. In the range of 40–60 °C, denaturation of proteins cause changes in quality parameters like water holding capacity (WHC) and texture [9].

Acid phosphatase (ACP, EC 3.1.3.2) is an enzyme that might be of interest in this context. ACP is similar to the far more documented alkaline phosphatase (ALP, EC 3.1.3.1) in activity behaviour. The thermal stability of ALP in milk is well documented, and has therefore been applied as an indicator for inadequate pasteurization for many years. The reported z-values of ALP range from 5 to 9.5 °C [10–14]. Similar to ALP, the ACP catalyses the hydrolysis of phosphoric mono ester bonds as well as trans localisation of phosphate groups. The true substrate is however not known. Minor variation in activity behaviour and structural characteristics (homo- or hetero-dimer, glycosylation) in different tissue as well as in different species indicate the existence of several iso-enzymes [15, 16]. ACP is localized in the lysozomes of the connective tissue primarily and widely distributed in the muscle of most species [17–19]. The specific role of ACP in fish is however not verified but higher activities found in the post-spawning hake suggest a potential relation to the change in muscle texture associated with spawning [20]. Similar phosphatase activities like ATP, ADP and IMP degrading enzymes, are also suggested to be related to freshness [21].

During investigation of ACP and ALP, Kuda et al. [17] found that the ACP activity was reduced (>90%) when 55 and 60 °C was applied for 10 min. Similar inactivation response of ACP was found in cooked broiler and turkey [22, 23]. However, neither of these studies presents kinetic data. Körmendy et al. [24] studied ACP in pasteurised ham, and reports a z-value of 6.94 °C. Explicit D-values were not given in this study, but ACP was presented as suitable for validating inactivation of *Streptococcus faecium* with a z-value of 10 °C and a pasteurising value at 70 °C of 80 min. ACP as a potential TTI in ground beef was also suggested by Orta-Ramirez et al. [25], with a z-value of 7.41 °C ($D_{68\,^{\circ}\mathrm{C}} = 3.33$ min) and the target organisms were *Escherichia coli* ($z = 5.6$ °C; $D_{68\,^{\circ}\mathrm{C}} = 0.12$ min) and *Salmonella seftenberg* ($z = 6.24$ °C; $D_{68\,^{\circ}\mathrm{C}} = 0.22$ min). However, other potential indicator enzymes were tested and triose phosphate isomerase was suggested to be used as TTI in beef products ($z = 5.56$ °C; $D_{66\,^{\circ}\mathrm{C}} = 0.74$ min).

The aim of this study was to document the inactivation kinetics of ACP in the extract of farmed cod during thermal processing. Further, the possible use of ACP as an intrinsic TTI for inactivation of micro-organisms and/or heat-induced changes in quality of the fish muscle will be discussed on the basis of knowledge of the enzyme inactivation kinetic parameters.

Material and methods

Raw material

Farmed Atlantic cod (*Gadus morhua*) ($n = 3$, male, approx. 2.8 kg) were obtained from the Norwegian Institute of Fisheries and Aquaculture Research, Tromsø, Norway (June 2005). Before slaughtering, the fish were starved for 19 days. The fish were killed by a blow to the head and gutted in a pre-rigor state (<5 h post slaughter) and subsequently sent overnight (on ice) to Norconserv AS, Stavanger, Norway. The fish were filleted approximately 24 h after slaughter.

Raw material characterization

The pH, water holding capacity (WHC), total protein, water and ash content (%) of the raw fillets were determined. The pH was measured using a pH meter (Orion 410Aplus Benchtop, Thermo Electron Corporation, MA, USA) equipped with a puncture combination electrode (Orion Ross 81–63, Thermo Electron Corporation, MA, USA). WHC was analysed gravimetrically using the method described by Varmbo et al. [26], but modified by using a stainless steel tube of Ø31 mm with filter 0.213 mm mesh (Patent No. 321375 B1, Norconserv, Stavanger, Norway). Total protein content was analysed based on the method of Kjeldahl [27]. The water and ash content was gravimetrically determined following drying for 16–18 h at 105 and 550 °C, respectively.

Sample preparation

A pooled sample (homogenate) of the dorsal muscle (loin, $n = 6$) was made in a Robot coupe blender (model no. R5A, Robot coupe S.A., Annee fab., Montceau en Borgogne, France). Parallel mince samples of 20 g were added to 1% Triton X-100 (1:9, w:w) (Sigma–Aldrich, Chemie GmbH, Steinheim, Germany) containing protease inhibitors (10 μM Leupeptin, 1 μM Bestatin and 1 μM Phenylmetylsulfonyl fluoride, Sigma–Aldrich Logistik, GmbH, Schelldorf, Germany) directly in centrifuge bottles (250 ml, Oak Ridge, Sorvall Centrifuges, Kendro Laboratory Products GmbH, Hanau, Germany) and subsequently blended with an Ultra Turrax (Model T 18 basic, IKA WERKE GMBH & CO.KG, Staufen, Germany) for 1 min (18 000 rpm/min). After blending, the samples were centrifuged at 2220 × g for 10 min at 3 °C (Sorvall RC-5C plus centrifuge with rotor SLA-1500, Kendro Laboratory Products GmbH, Hanau, Germany). The sample extracts (supernatants) were pooled and collected in tubes (50 ml). All handling and preparation of the samples

were performed on ice. The sample extracts were frozen (-80 °C) until heat treatment and analyses of the enzyme (ACP) activity.

Heat treatment of sample extracts

The individual aliquots of frozen sample extract were thawn in cold water before heat treatment. Sample extracts (0.5 ml) were incubated (55.0, 57.5, 60.0, 62.5, 65.0 or 67.5 °C) in duplicate in glass tubes (Ø12 mm) in a water bath (Hetotherm, Heto Lab Equipment, Denmark) at different time intervals (2–60 min, depending on the isothermal temperature). The tubes were sealed by rubber stoppers during the heat treatment. A FISO workbench (FISO Technologies Inc., Quebec, Canada) with fibre-optic cables, loggers and software were used to record the temperature profile in the individual tubes. The tubes were immersed in an ice bath after heat treatment for immediate chilling.

Phosphatase activity assay

The ACP activity was analysed according to the method of Kuda et al. [17] for both heat-treated samples and untreated samples. After centrifugation ($2220 \times g$, 10 min) the supernatant of the extracts were applied (25 μl) in quadruplicate to a multi-well plate (Greiner bio-one GmbH, Frickenhausen, Germany). The substrate solution containing 15 mmol/l PNP (4-nitrophenyl phosphate di-sodiumsalt hexahydrate, Fluka, Sigma–Aldrich Chemie GmbH, Schnelldorf, Germany) and 1 mmol/l $MgCl_2$ (magnesium chloride, MERCK, Hoenbrunn, Germany) dissolved in sodium acetate, pH 5.5 (MERCK, Damstadt, Germany), was added (150 μl) to the multi-well plate. Subsequently, the samples were incubated at 37 °C in a heat cabinet before measurement of the ACP activity. The signal measured with 5 min intervals (8 parallels) at 405 nm increased linearly with the incubation time in the range up to 60 min. The incubation time were set to 30 min at 37 °C for all samples. The enzyme–substrate reaction was quenched by addition of NaOH (50 μl, 0.5 mol/l, Riedel-de Haen AG, Seelze, Germany).

Blank samples (extract replaced by distilled water) were treated according to the same procedure and used as controls. The average absorbance of the blank at pH 5.5 (405 nm) was 0.2, or approximately $A = 0.05$ (Abs/min/g). The ACP activity (A_{ACP}) was measured as the absorbance of p-nitrophenol (405 nm) released from the substrate in a micro-plate reader (ELx800, Bio-Tek Instr. Inc., Winooski, USA; KC Junior software, Bio-Tec Instrumentation Inc., Winooski, USA).

By measuring ACP in extract diluted to infinity, the absorbance was confirmed to be Abs = 0.2 (Abs sample = Abs blank) and this was used as a limit in the calculated ACP activity.

Calculations

ACP values

The ACP activity in the samples was expressed as the absorbance difference (sample – blank) per minute of incubation (30 min) per gram of muscle sample in the extract.

$$A = \left[\frac{df(\mathrm{Abs_s} - \mathrm{Abs_b})V_{\mathrm{w}}}{(V_{\mathrm{e}} t_{\mathrm{i}})}\right] \quad (1)$$

where A = absorbance of p-nitrophenol at 405 nm (Abs/min/g sample), $\mathrm{Abs_s}$ = absorbance of sample at 405 nm, $\mathrm{Abs_b}$ = absorbance of blank at 405 nm, V_{w} = total volume in well (sample extract + substrate, 225 μl), V_{e} = volume of sample extract (25 μl), t_{i} = incubation time (30 min), df = dilution factor (9).

D and z-values

The remaining ACP activity ($\mathrm{ACP_r}$) after thermal treatment was presented as "residual activity" according to Eq. (2):

$$\mathrm{ACP_r} = \left[\frac{A}{A_0}\right] \quad (2)$$

where ACP_{r} = residual ACP activity, A = enzyme activity of heat-treated sample (at time t), A_0 = initial enzyme activity of sample not subjected to heat treatment.

Subsequently, the calculation of the decimal reduction time (D-value) was performed from log transformed data of residual activity ($\mathrm{ACP_r} = A/A_0$) based on the Thermal Death Time Concept typical for first-order reactions in foodstuffs as described by several authors [28, 29]. Linear regression was done in Minitab 14.13 (Minitab Inc., PA, USA).

The D-values were plotted against temperature and a log linear regression curve was used to determine the z-value, i.e. the temperature increase necessary for 10-fold decrease in the D-value.

Results

Characterisation of the raw material

The pH and WHC of the fillets ($n = 3$) 1 day post mortem was $6.15 \pm 0.04\%$ and $88.0 \pm 3.2\%$, respectively. The total protein content in the pooled sample of loins was 19.1 g/100 g and water and ash content 79.1 g/100 g and 1.28 g/100 g, respectively. The ACP activity in the raw material extracts was 2.52 ± 0.11 Abs/min/g sample.

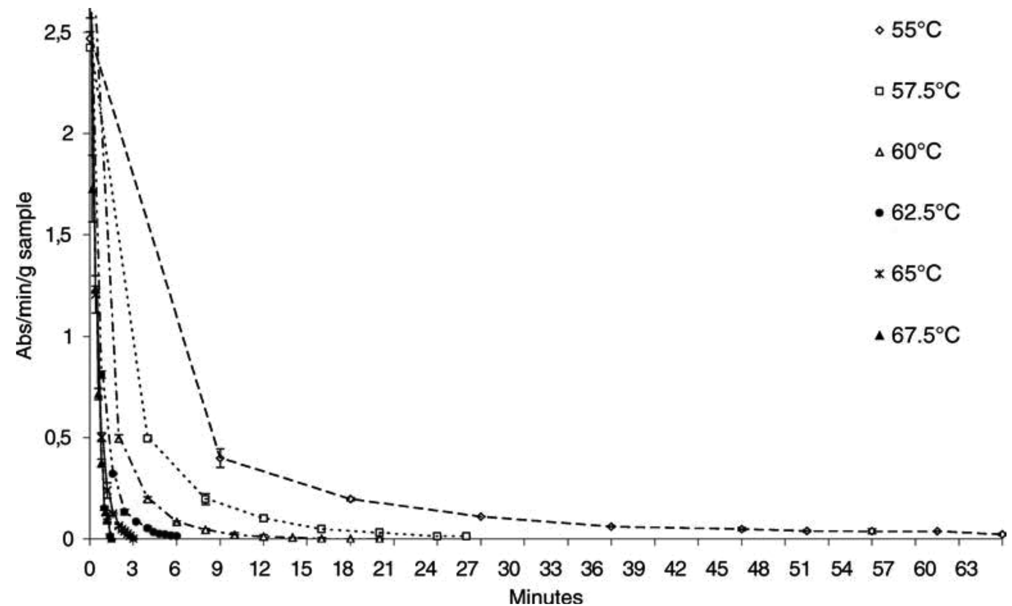

Fig. 1 The effect of process time and temperature on the ACP activity in extracts of cod muscle

Effect of heat treatment on the ACP activity

Temperature in the water bath was relatively stable during immersion of the glass tubes (± 0.5 °C). The temperature in the glass tubes increased rapidly when immersed in the water bath. The time to reach the set temperature ($n = 3$) was 53 ± 1 s for isothermal heating at 40 °C and 57 ± 6 s for the isothermal heating at 60 °C. Cooling in ice water resulted in a very fast drop below 20 °C.

The activity of ACP in the sample extracts was significantly decreased with increasing heating time (1–60 min) and temperature (55–67.5 °C) (Fig. 1). When exposing the extracts to a thermal load in the interval 62.5–67.5 °C, the measured ACP activity decreases rapidly and was below the blank value of 0.05 (Abs/min/g) within less than 4 min. Thus, no enzyme activity remained after a short heat treatment time at the higher temperatures due to inactivation or degradation. A further decrease in the thermal load (60.0, 57.5 or 55.0 °C) significantly increased the time before heat-inactivation of the enzyme is reached. At 60 °C the enzyme activity was below the detection limit after approximately 12 min, but at 55 °C some residual activity was still detectable even after 60 min of treatment.

Kinetic parameters

The decimal reduction time (D-value) was calculated based on the slope of a fitted curve (linear regression) to the ACP_r-values ($\log (A/A_0)$) at the investigated temperatures (Table 1). The linear regressions between the ACP_r-values and heat treatment time at the different temperatures indicate that the inactivation of the enzyme follows a first-order model. The D-value decreases with increasing temperature in the investigated interval (Table 1). The adjusted coefficients of multiple determinations are greater than 0.90 for all measured temperatures except for 65 °C (0.759). A linear regression of the log transformed D-values and the heat treatment temperature was obtained and presented in Fig. 2. The coefficient of multiple determinations for the regression curve was 0.988.

Table 1 D-values (min) obtained by linear regression between the log ACP_r-values and heat treatment temperature (55.0–67.5 °C) with correlation factors and standard error

Temperature (°C)	D-value (min)	R^2	Standard error	Number of data (n)
55.0	47.62	0.945	0.099	25
57.5	13.48	0.987	0.071	16
60.0	6.05	0.929	0.185	19
62.5	3.22	0.978	0.068	16
65.0	1.36	0.759	0.239	28
67.5	0.38	0.949	0.127	10

The regression line in Fig. 2 was used to obtain the z-value. The z-value for ACP in the sample extracts was 6.37 ± 0.09 °C. Thus, a temperature increase of 6.37 °C is required to obtain a 10-fold decrease in the D-value.

Discussion

Residual ACP activity in cod after heat inactivation showed a log linear decay with time when heated at isothermal conditions in the range of 55–67.5 °C for 1–60 min obtaining $D_{60} = 6.78 \pm 0.10$ min and $z = 6.37 \pm 0.09$ °C. The residual ACP was below the level of the blank within the time frame used for each temperature. Under all conditions

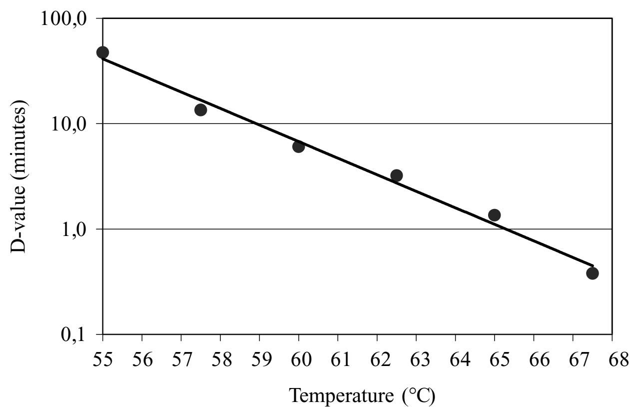

Fig. 2 *D*-values for acid phosphatase obtained in cod muscle extract plotted against temperature and regression line ($R^2 = 0.988$)

studied, first-order reaction kinetics was observed. This is in contradiction with earlier observations in pasteurized ham as presented by Körmendy et al. [24] who concluded that the inactivation was not of first-order and also measured residual ACP activity after 600 min heating at 65 °C. However, in spite of this conclusion, Körmendy et al. [24] calculated a constant *z*-value of 6.94 °C in the range of 60–80 °C, which is in the same order of magnitude as found in our study. Orta-Ramirez et al. [25] studied ACP among several other enzymes in ground beef and found a *z*-value of 7.41 °C and a *D*-value of 3.33 min at 68 °C. The obtained *D*-values in this study were much lower, but higher than reported by Kuda et al. [17].

From the regression curve for the *D*-values in the range of 55–67.5 °C, a *D*-value at 70 °C (D_{70}) of 0.18 min for the ACP could be extrapolated by using a *z*-value of 6.4. It may be possible to measure up to 1.5 log inactivation of the enzyme, i.e. a pasteurisation value of $P_{70}^{6.4}$ of 0.27 min. The *D*-value of the enzyme was in the same order of magnitude as the *D*-value of *Listeria monocytogenes*, D_{70} is 0.33 min [6], but not suitable for monitoring a 6 log inactivation which would equal a pasteurisation value $P_{70}^{7.5}$ of 2 min. In addition, the *z*-value was lower than reported by ECCF [6] which was 7.5. There is, however, a wide range of kinetic data reported for *L. monocytogenes* depending on the strain the model system used for determining the heat resistance [30]. For cod, Ben Embarek [30] investigated the heat resistance of *L. monocytogenes* O62 and O57 and found D_{70} of 0.03 min (*z*-value 5.7) and D_{70} of 0.05 min (*z*-value 5.7), respectively. According to this, a 6 log inactivation would require a pasteurisation value P_{70} in the range of 0.18–0.30 min. The *z*-values of the two strains of *L. monocytogenes* were however slightly lower than the *z*-value of ACP and limit the possible use of the enzyme for mimicking inactivation of these strains. ACP in cod extracts treated with protease inhibitor was therefore not suitable as an intrinsic quantitative TTI for monitoring heat-resistant pathogenic bacteria in cod.

Heat-induced quality changes in cod related to denaturation of proteins are well known. Kinetic parameters of the proteins in cod should therefore be found, and compared to the kinetic parameters of ACP activity. However, seasonal variation of the ACP (and other biological factors) should be examined before any application of an intrinsic quantitative TTI. The biological values of farmed cod presented here have been reported at similar values by other authors [6]. However, the awareness of biological differences (wild, cultured and seasonal) is important during the comparison of kinetic data.

Conclusion

Thermal inactivation of acid phosphatase activity in medium with protease inhibitor was measured in the range of 55–67.5 °C and calculated to $D_{60} = 6.78 \pm 0.10$ min and $z = 6.37 \pm 0.09$ °C. These data show that ACP is less thermostable than heat-resistant pathogen micro-organisms (e.g. *Listeria monocytogenes*) and therefore could be inactivated in heat-preserved fish products. As a consequence, ACP residual activity is not recommended as a quantitative intrinsic TTI based on our study in an extract model of cod.

Further validation of the inactivation behaviour of ACP in fish samples is required in order to confirm that the ACP enzyme will be inactivated in a commercial pasteurisation process as well, or to prove that ACP residual is not a shelf life limiting factor of heat-preserved fish products. However, the results from the extract model will be a nice guideline during this study.

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Paper IV

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Use of Residual Acid Phosphatase Activity in Heat-Processed Atlantic Cod (*Gadus morhua*) for Estimating Thermal Load

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ABSTRACT

Farmed Atlantic cod muscle tissue was heated isothermally at temperatures of 56 to 68°C for 15 s to 25.5 min. Extracts from the heat-treated samples were prepared by mixing with Triton X-100 (1:9, wt/wt) and subsequent centrifugation. Residual acid phosphatase (ACP) activity was measured, and the inactivation was modeled in two phases. Mean (±standard error) kinetic parameters of thermal inactivation were determined as $D_{60^\circ C} = 34.93 \pm 2.02$ min and $z = 22.01 \pm 4.10$°C for short heating times and $D_{60^\circ C} = 3.19 \pm 0.11$ min and $z = 6.31 \pm 0.51$°C for heating times longer than 100 s. The data support the use of residual ACP activity for modeling a 6-log inactivation of *Listeria monocytogenes* at 60°C but only a 4-log inactivation at 70°C. Extracts prepared from raw muscle and subsequently heated isothermally at temperatures of 54 to 70°C for 2 to 51 min were used to obtain kinetic parameters $D_{60^\circ C} = 7.98 \pm 1.11$ min and $z = 6.92 \pm 0.07$°C. A short initial drop in ACP activity was observed in raw cod muscle during freezing and cold storage. Subsequently, the activity was stable for 24 months. ACP activity in raw cod muscle did not seem to be influenced by gender, season, or brining with different levels of salt and phosphates. No other factors that could inactivate ACP were found, and usual activity of 2.54 ± 0.02 Abs/(min*g sample) can be expected in frozen and thawed farmed cod muscle.

During recent years, obstacles to farming of various fish species, e.g., cod (*Gadus morhua*), have been solved *(12, 28),* and cod has received much attention from the fast-growing aquaculture industry *(18, 21, 24, 25).* The increasing number of species available for aquaculture and an increasing market demand for convenience fish products are elements stimulating this market sector, and mild heat processing (in the range of 60 to 75°C) of vacuum-packed cod loins has been implemented commercially *(26).* One of the challenges to fish processing is establishment of an appropriate thermal load that will destroy pathogenic and spoilage microorganisms and enzymes *(1, 19)* while maintaining the sensory attributes characteristic of freshly prepared fish. Thermal inactivation of enzymes is important because enzymes may limit product shelf life. However, thermostable enzymes could act as an intrinsic biological marker; the amount of enzyme activity remaining could be used to determine the heat load applied to the product. Residual acid phosphatase (ACP) activity has previously been suggested as an intrinsic time-temperature integrator (TTI) for monitoring thermal inactivation of heat-resistant pathogenic microorganisms in several products (e.g., ham and turkey) *(14).* Thermal inactivation of ACP (EC 3.1.3.2) in cod extracts has been reported to be inadequate as a quantitative intrinsic TTI because of the low heat resistance of ACP compared with that of relevant target pathogenic microorganisms (e.g., *Listeria monocytogenes*) *(13).* However, in the same study, further investigations on in situ inactivation kinetics and extracts without addition of protease inhibitor were suggested. Knowledge of thermal inactivation kinetics of ACP also could be relevant with respect to quality degrading enzymes, which can be expected to have a heat resistance in the same order of magnitude as that of ACP *(3).*

The aim of this study was to investigate the potential of ACP as an intrinsic biological marker for heat inactivation by studying the correlation between ACP activity and microbiological safety and to investigating parameters that could affect ACP content (e.g., seasonal variation and fish gender) and thermal resistance (e.g., brining). Using a large batch of muscle from male and female cod, this study extends our previous work *(13)* and includes the ACP thermal inactivation kinetics in both extracts and muscle tissue before extraction to document the potential use of both types of ACP information.

MATERIALS AND METHODS

Raw material. Farmed Atlantic cod (94 individuals) were obtained from an aquaculture research station (Havbruksstasjonen AS, Skulgambukt, Tromsø, Norway). Fish samples were from the same batch as used in our previous study *(27),* in which several parameters were reported for the raw material. The pooled samples of a mixture of male and female fish were used for the kinetic study and the study on influence of additives on ACP stability.

For the seasonal study, mature farmed male and female Atlantic cod (Havbruksstasjonen AS) with an average live weight of 2.8 kg were kept in sea cage from February 2005. The fish were fed a dry pelleted feed (BioMar, Myre, Norway) and starved for 19 days in 4°C water before slaughter in December 2005. In

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addition to the initial harvest, seven individuals of random gender were caught at eight occasions during the following year (February, March, May, June, September, October, December 2006 and March 2007). The fish were killed by a blow to the head and then exsanguinated in seawater for 30 min after the isthmus was cut. The fish were gutted before rigor mortis within 5 h of slaughter and kept on ice until preparation of samples. For all seasons (except December 2005 and February 2006), the ACP activity was measured twice, but no significant difference in activity was found at 7 and 13 days after slaughter. During the seasonal study, no analyses were made immediately after slaughter (before rigor mortis).

Preparation of sample extracts. A pooled homogenated sample of the dorsal muscle (loin, $n = 6$) was made in a blender (model R5A, Robot Coupe S.A., Montceau en Borgogne, France). Parallel ground samples of 20 g were added to 1% Triton X-100 (1:9, wt/wt; Sigma Aldrich Chemie GmbH, Steinheim, Germany) directly in centrifuge bottles (250 ml; Oak Ridge, Sorvall Centrifuges, Kendro Laboratory Products GmbH, Hanau, Germany) and subsequently blended in an Ultra Turrax homogenizer (model T 18 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany) for 1 min (18,000 rpm/min). After blending, the samples were centrifuged at 2,220 $\times$ g for 10 min at 3°C. The sample extracts (supernatants) were pooled and collected in 50-ml tubes. All sample handling and preparation steps were performed on ice. The sample extracts were frozen at −80°C until heat treatment and analysis of ACP activity.

Heat treatment of sample extracts. To use ACP activity as a TTI for impact evaluation, the kinetics of heat inactivation of ACP in extracts must be understood. The heat stability of ACP was examined outside the muscle matrix, and the ACP activity was measured as a function of treatment time and temperature. Individual aliquots of frozen sample extract were thawed in cold water before heat treatment. Sample extracts (0.5 ml) were incubated in triplicate in glass tubes (12-mm diameter) at 2°C intervals from 54 to 70°C in a water bath (Hetotherm, Heto-Holten Lab Equipment A/S, Allerod, Denmark) at different time intervals (2 to 51 min depending on the inactivation temperature). The tubes were sealed with rubber stoppers during the heat treatment. Fiber-optic cables, logger. and software (FISO Technologies Inc., Quebec, Canada) were used to record the temperature profile in the individual tubes. The tubes were immersed in an ice bath after heat treatment for immediate chilling to stop further inactivation of ACP.

Heat treatment of cod muscle. To use ACP activity as a TTI for impact evaluation in cod, the kinetics of heat inactivation of ACP in situ must be understood and compared with that in extracts. Therefore, a kinetic study of the heat inactivation of ACP was performed in a manner similar to that in extract. Ground cod muscle tissue (3.29 $\pm$ 0.13 g, 4 mm thick) was weighed into test cells (31-mm internal diameter) designed for rapid heating with good temperature distribution within the sample ($n = 4$) (Hettich GmbH & Co., Tuttlingen, Germany). The test cells were sealed and immersed in a water bath with circulation (Heto-Holten) for isothermal heat treatment of the samples at temperatures of 56, 60, 64, or 68°C) at time intervals of 1.6 to 25.5 min (depending on the temperature). After heat treatment, the test cells were immersed in an ice bath for immediate chilling. Extracts of the cooked muscle samples were prepared as described above. The individual sample extracts (supernatants) from cooked muscle were analyzed for ACP activity. Temperature was measured in the center of one fish sample at each temperature during heating and cooling with fiber-optic sensors (FISO).

Brine. To assess the effect of salt and brining on ACP activity and heat stability, seven brines were blended with ground fish muscle in a ratio of 1:5 and incubated for 3 h at 4°C before analysis.

All brines were prepared from salt (1.5 g NaCl, Akzo Nobel, Refined Salt, Dansk Salt AS, Mariager, Denmark) and/or phosphate compound (1.5 g), and distilled water was added to a total volume of 50 ml to make a 3% (wt/vol) solution, except for one solution in which only 1.5% NaCl was added. The phosphate compounds were all purchased from Fluka GmbH (PM Custom-Ware, Buchs, Switzerland) and were of puriss or purum grade (mono-phosphate [$Na_2HPO_4 \cdot 2H_2O$] and basic diphosphate [$Na_4P_2O_5 \cdot 10H_2O$], assay $\geq$ 99%; tripolyphosphate [$Na_5P_3O_{10}$], assay $\geq$ 98%) except the hexametaphosphate ([$NaPO_3$]12-13·Na_2O, assay 65 to 70% P_2O_5 basis).

Liquid (water or brine, 4 ml) was mixed into the ground fish tissue (16 g), to make approximately 0.6% added salt and phosphate compounds in samples. This concentration equals a P_2O_5 content of 2.39 to 4.18 g/kg of cod, depending on the relative amount of phosphate in the different compounds used in the brines and is within acceptable limits of additives in fish (P_2O_5 at 5 g/kg of sample for total added phosphates) *(9)*. The ACP activity was determined on the extracts as described above both before and after a predefined heat treatment. A constant time and temperature study revealed an increase in ACP activity up to 50°C, at which point inactivation occurs. The heat treatment was performed in the water bath as described above but under constant incubation conditions (50°C for 10 min). The ACP activity in extracts of brined sample was compared with that in extracts of samples treated with water to investigate the influence of specified additives (brine components) in cod samples. The effect on the ACP activity of adding salts to the brine were tested by analyzing residual ACP in the extracts of samples with salt or with both salt and phosphates before and after heat treatment.

ACP activity assay. ACP activity was analyzed according to the method of Kuda et al. *(16)*. The cooked extracts or extracts from cooked muscle (25 μl) were added in quadruplicate to a multiwell plate (Greiner bio-one GmbH, Frickenhausen, Germany). A substrate solution (150 μl) containing 15 mM PNP (4-nitrophenyl phosphate disodium salt hexahydrate; Fluka, Sigma-Aldrich) and 1 mM $MgCl_2$ (Merck, Hoenbrunn, Germany) dissolved in 0.1 M sodium acetate buffer, pH 5.5 (Merck) was added to the multiwell plate, and samples were incubated in a heating cabinet before measurement of the ACP activity. Before quenching, the signal increased linearly with increasing time up to 60 min (measured at 5-min intervals, eight parallels), as occurs in other fish species *(16)*. An incubation time of 30 min at 37°C was selected for residual ACP activity assessment. The enzyme-substrate reaction was quenched by addition of 50 μl of 0.5 M NaOH (Riedel-de Haen AG, Seelze, Germany). The signal was measured at 405 nm in a microplate reader (Synergy 2 and software, Bio-Tek Instruments Inc., Winooski, VT).

The ACP activity (A) was measured as the absorbance of p-nitrophenol released from the substrate under the assay conditions:

$$A = \left[\frac{df \cdot (\mathrm{Abs}_s - \mathrm{Abs}_b) \cdot V_w}{(V_e \cdot t_i)}\right] \quad (1)$$

where A is the absorbance of p-nitrophenol at 405 nm (Abs·[min·g sample]$^{-1}$), Abs_s is the absorbance of sample at 405 nm, Abs_b is the absorbance of blank at 405 nm, V_w is the total volume in the well (225 μl: 25 μl of sample extract, 150 μl of substrate, and 50 μl of NaOH), V_e is the volume of sample extract (25 μl), t_i is the

incubation time (30 min), and *df* is the dilution factor (10 ml of extract per g of muscle).

Blank samples (extract replaced by 25 μl of distilled water) were treated according to the same procedure and used as controls to account for spontaneous hydrolysis of the substrate. The detection limit was determined by serial dilution of extract until the absorption of the sample equaled the absorption of the blank at 405 nm (at approximately 30-fold dilution of the original extract containing a mean of 2.5Abs/(min·g). Further dilution produced overestimation of results, and the detection limit of residual ACP was set accordingly to $A = 0.08$ Abs/(min·g).

The significance of age, gender, and type of brine was determined by an analysis of variance (ANOVA) general model and a Tukey test, using the software Minitab 14.13 (Minitab Inc., State College, PA).

Kinetic data analysis. Heat inactivation of enzymes can often be described by a first-order reaction (equation 2):

$$\frac{dX}{dt} = -kX \tag{2}$$

where A is the residual enzyme activity at time t and k is the inactivation rate constant (per minute). In case of isothermal experiments (i.e., rate constant k not varying with time), equation 2 can be integrated, obtaining equation 3, where A_0 is the activity at 0 min of the isothermal treatment:

$$A = A_0 \cdot e^{(-kt)} \tag{3}$$

In the area of food science and technology, it is common to characterize first-order reactions using the thermal death time concept. The decimal reduction time (D-value) is the time needed for a 90% reduction of the response value at a given constant temperature. For a first-order reaction, D-values and rate constants are inversely related:

$$D = \frac{\ln(10)}{k} \tag{4}$$

Substitution of equation 4 into equation 3 yields an alternative equation for a first-order reaction:

$$A = A_0 \cdot 10^{-\frac{t}{D}} \tag{5}$$

The validity of a first-order inactivation was examined by plotting residual enzyme activity (ACP_r) defined in equation 6 versus treatment time on a semilogarithmic scale and by evaluation of the correlation parameter R^2:

$$ACP_r = \left[\frac{ACP_t}{ACP_u}\right] \tag{6}$$

where ACP_r is the residual ACP activity and ACP_u is the enzyme activity of untreated sample.

In the thermal death time model, the temperature dependence of the D-value is given by the z-value (equation 7), which is the temperature increase necessary to obtain a 10-fold decrease of the D-value:

$$D = D_{ref} 10^{(T_{ref} - T)/z} \tag{7}$$

where D_{ref} is the decimal reduction time at the reference temperature (T_{ref}), and z is the z-value. The D-values were plotted against temperature with Minitab 14.13 (Minitab Inc., State College, PA), which generated a log linear regression curve used to determine the z-value. Because this approach involves the use of the results obtained from a first regression (D-value determination) in a second regression (z-value determination), the errors on the estimates of the first regression are not taken into account in the second model. This underestimation of the error in z-values could have been avoided only by using nonlinear regression, which was not available in the software.

For practical use, a pasteurization value P (or F), based on the kinetic parameters of heat inactivation, is commonly used and can be calculated as shown:

$$P^z_{T_{ref}} = \int_0^t 10^{(T_c - T_{ref})/z}\, dt \tag{8}$$

A biphasic behavior, where the regression lines for estimating D-values are broken, can sometimes be observed *(30)*. For estimating the kinetic parameters of ACP activity in extracts from heat-treated cod meat, inactivation was divided into a heat-labile phase with parameters $D_{L,ref}$ and z_L and a heat-stable phase with parameters $D_{S,ref}$, z_S, and A_{0S} of the biphasic model. The kinetic parameters were determined for each phase as described above for one phase.

Deviations in temperature during the experiment were corrected for by using equation 7 to determine a correction factor and by repeating the calculation in an iterative process.

RESULTS AND DISCUSSION

Factors affecting ACP activity and stability in cod muscle. The ACP activity (mean ± standard error [SE]) in the frozen and thawed raw material extracts from the pooled sample (mixed gender, $n = 70$) was 2.54 ± 0.02 Abs/(min·g sample), which is very close to the findings in our previous work *(13)*. This value is significantly below the ACP activity in fresh (not frozen) extracts of 3.20 ± 0.09 Abs/(min·g sample) ($n = 3$). However, after the initial drop due to freezing and thawing, the ACP activity did not change during 24 months of storage at −80°C.

The ACP activity (mean ± SE) in the frozen and thawed muscle extracts from the pooled sample of each gender ($n = 3$) was 2.63 ± 0.11 Abs/(min·g) for male fish and 2.64 ± 0.11 Abs/(min·g) for female fish, respectively. There was no significant difference ($P = 0.933$) in ACP activity for raw material with respect to gender.

The initial level of ACP activity in raw cod muscle at each harvest is shown in Table 1. Significant differences in ACP activity were not consistent and could be attributed to stress-related events (e.g., temperature changes or spawning) as reported by other researchers for a wide range of raw materials *(5, 6, 23)*, although these studies involved ACP (or alkaline phosphatase) response in tissue organs (liver, gills, or gonads) or direct measurement of blood samples.

ACP activity in muscle extracts from pooled samples of mixed gender with NaCl (0.3 and 0.6% in sample) and other brine additives is shown in Table 2. In cooked muscle, added brine of 1.5% NaCl (0.3% in sample) had no significant effect on ACP activity ($P = 0.0001$). Addition of NaCl to the fish muscle can be expected to have an influence on ACP activity in raw material but not for the cooked material. Hence, addition of NaCl at up to 0.6% in the sample does not influence the feasibility of ACP as an intrinsic biological marker for heat load on the product.

Raw extracts with added phosphates did have significantly reduced A_u compared with samples with no additives. However, when extracts were isothermally heated at 50°C for 10 min, no significant difference in ACP activity

TABLE 1. *ACP activity in raw cod extracts as affected by harvest time*[a]

Time of harvest	No. of samples[b]	ACP activity (Abs/[min·g]) Mean[d]	Standard error	Standard deviation	Milt wt (% of fish wt)[c] Mean	Standard deviation	Mean temp (°C)
December 2005	16	2.77 AB	0.04	0.16	12.00	2.68	6.9
February 2006	7	2.74 AB	0.10	0.26	24.77	4.74	4.5
March 2006	14	2.60 A	0.09	0.33	16.18	2.22	3.9
May 2006	28	2.87 AB	0.07	0.35	3.87	3.79	4.5
June 2006	27	2.98 B	0.06	0.29	0.96	0.49	7.0
September 2006	27	2.76 AB	0.10	0.49	0.63	0.06	9.9
October 2006	28	2.65 ACD	0.05	0.29	2.82	1.39	8.0
December 2006	28	2.49 CD	0.05	0.26	13.12	1.87	5.8
March 2007	21	2.59 ACD	0.07	0.31	16.87	2.19	3.6

[a] All (mature) fish were kept in sea cage from February 2005.
[b] Number of samples is not the same as number of individual fish; several samples were obtained from each fish.
[c] Milt weight is included as an indicator for maturation of the fish.
[d] Means with the same letter are not significantly different ($\alpha = 0.05$). Significance level was based on individual 95% confidence intervals for means based on the pooled standard deviation calculated by the general linear model ANOVA.

was found. Thus, ACP activity in cooked samples is not expected to be influenced by addition of phosphates to the levels used in this study.

Kinetic study on heat inactivation of ACP. Heat inactivation of ACP in cod extracts is shown in Figure 1. The decimal reduction time (*D*-value) was calculated based on the slope of the fitted curve (linear regression) of the ACP_r values ($\log[A/A_0]$) versus time at the temperature investigated (Table 3). The *D*-value decreased with increasing temperature in the investigated interval (Fig. 2). The correlation coefficients (R^2) of multiple determinations are greater than or equal to 0.95 for all measured temperatures. A linear regression ($R^2 = 0.992$) was performed on the log-transformed *D*-values and the heat treatment temperature (Fig. 2).

The regression line in Figure 2 was used to obtain the *z*-value (6.92 ± 0.07°C) for inactivation of ACP in the sample extracts. Based on the *z*-value and the observed *D*-values at each temperature, the $D_{60°C}$ was 7.98 ± 1.11 min and the $D_{70°C}$ was 0.29 ± 0.04 min.

A direct comparison of kinetic data for ACP obtained from different biological material and by procedures other than those in the present study is difficult, but an overview of some findings is given in Table 4. Both *z*- and *D*-values were close to our previous findings *(13)* obtained for extracts from the muscle of three male cod from the summer season with added protease inhibitor.

Heat inactivation of ACP in cod muscle. The time-dependent changes in ACP activity during heat treatment are shown after correcting for dynamic temperature profile during the first 1 to 2 min of heating (Fig. 3). The heat inactivation of the enzyme activity can be divided in two phases. In the first phase from the start of heat treatment up to approximately 90 s there is rapid inactivation, whereas the heat inactivation at longer treatment times (>120 s) is much slower. In both phases a log-linear regression could be

TABLE 2. *ACP activity of pooled samples of brined raw cod extracts and isothermally heated cod extracts from male and female fish with combinations of NaCl and additives*

Additives	*n*	Mean ± SD ACP activity (Abs/[min·g])[a] Raw extracts	Heated extracts[b]
None	6	2.51 ± 0.10 A	1.26 ± 0.04 D
0.6% NaCl	2	2.45 ± 0.03 A	1.28 ± 0.00 D
0.3% NaCl	8	2.07 ± 0.02 B	1.17 ± 0.07 D
Water	2	2.60 ± 0.07 A	1.29 ± 0.06 D
0.6% diphosphate (alkaline) + 0.6% NaCl	8	2.29 ± 0.06 C	1.14 ± 0.06 D
0.6% diphosphate (acidic) + 0.6% NaCl	7	2.06 ± 0.06 B	1.25 ± 0.14 D
0.6% hexametapolyphosphate + 0.6% NaCl	4	2.51 ± 0.03 A	1.10 ± 0.05 D
Monophosphate + 0.6% NaCl)	5	2.21 ± 0.09 BC	1.16 ± 0.07 D
Triphosphate + 0.6% NaCl	4	2.62 ± 0.20 A	1.19 ± 0.04 D

[a] Means with the same letter are not significantly different ($\alpha = 0.05$). Significance level was based on individual 95% confidence intervals for means based on the pooled standard deviation calculated by the general linear model ANOVA.
[b] Heated at 50°C for 10 min.

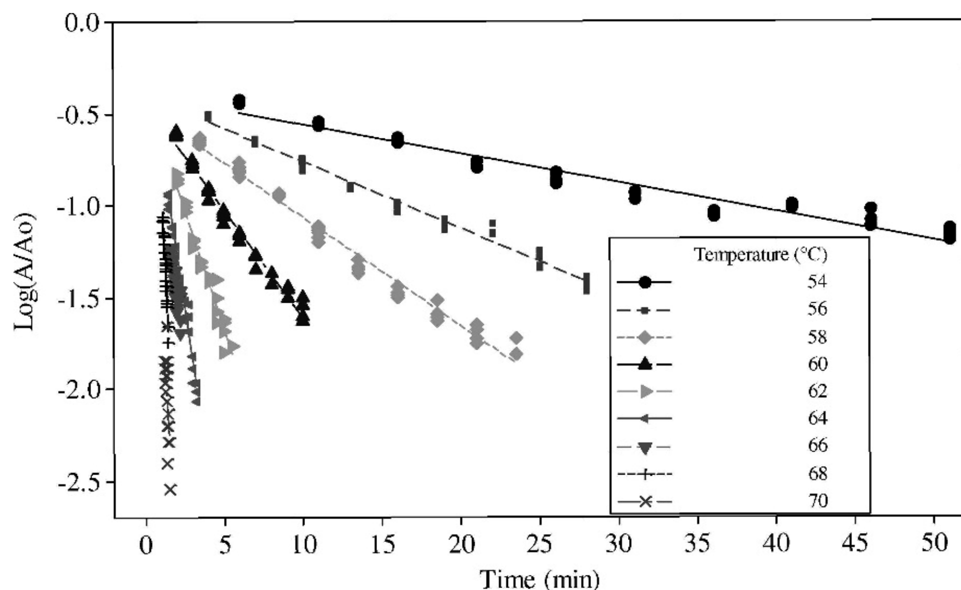

FIGURE 1. *Effect of process time (minutes) on the residual ACP activity in extracts of female and male ground cod muscle heated at 54°C (●), 56°C (■), 58°C (◆), 60°C (▲), 62°C (▶), 64°C (◀), 66°C (▼), 68°C (+), and 70°C (×).*

used with an acceptable fit (Table 4). The residual enzyme activity (A) may be predicted at different combinations of time and temperature by equation 5, using the D_L-values for short heat treatment and the D_S-values for treatment times above the shift time given in Table 4. Our interpretation of these results is that the enzyme response to heat can be divided in two parts: one that is heat labile and one that is more heat stable. During the first 1 to 2 min of heat treatment, the heat-labile part is inactivated to a level where it cannot be detected. D-values for inactivation at heating times above 2 min is considerably higher than that for heat-labile inactivation, but the difference decreases with increasing temperature, which means that the heat-labile part becomes increasingly important at higher temperatures.

Heat processing time of a portion of vacuum-packed cod loin in a retort or steam cabinet starting from chilled and heating to 69°C would usually be 10 to 30 min. For a cod loin of 100 by 60 by 24 mm (127 g), the time to increase the core temperature from 4 to 69°C in a steam cabinet of 75°C would be a minimum of 22 min. This 22-min heating time would result in a 6-log inactivation of *L. monocytogenes* ($P_{70°C}^{7.5°C}$ = 2 min) in the core of the fish, a 4-log reduction of the heat-labile portion of the enzyme, and a 1.7-log reduction of the heat-stable enzyme activity. Thus, any remaining heat-labile enzyme activity in this example cannot be detected with the methods used here, which means that all of the remaining ACP activity would be from the heat-stable part.

The most challenging aspect involving the use of ACP as a TTI is to determine the remaining enzyme activity at the shift time. Further research into this activity could be made by using short preheating and rapid cooling before a second heat treatment and analysis of the fish muscle before, between, and after the two experiments.

The temperature dependence of the D-values was accurately described by equation 7 and D-values from Table 4, and the mean (±SE) z-value was 22.01 ± 4.10°C for the heat-labile portion of the ACP activity by linear regression (R^2 = 95.3) and 6.31 ± 0.51°C for heating periods longer than the shift time (R^2 = 98.9). The reference D-values determined at 60°C from the regression lines were 34.93 ± 2.02 min and 3.19 ± 0.11 min, respectively.

Comparison of the D-values in Table 4 clearly reveals that ACP is more heat stable in cod muscle than in the extract; at the same temperature higher D-values were observed. Even though ACP in cod muscle was inactivated

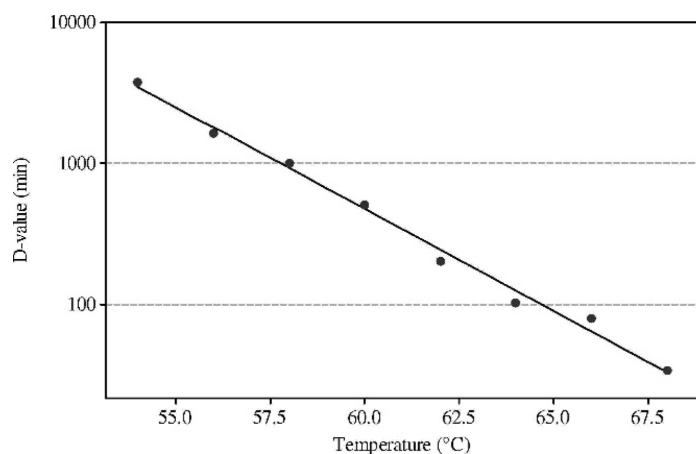

FIGURE 2. D-*values for first-order ACP inactivation in female and male ground cod muscle extract as a function of temperature. Full line represents the fitted values based on equation 7 (*R^2 = *0.992) determined for the heat-stable part of the enzyme.*

TABLE 3. *Estimated* D-*values of first-order isothermal inactivation of ACP in cod muscle extract determined by linear regression analysis for the heat-stabile part of the enzyme*

Temp (°C)	*D*-value (min)		
	Mean	SE	R^2
54	63.13	0.19	0.953
56	27.41	0.04	0.985
58	16.84	0.05	0.983
60	8.46	0.05	0.976
62	3.41	0.06	0.976
64	1.70	0.06	0.983
66	1.32	0.06	0.945
68	0.57	0.06	0.951

TABLE 4. *Estimated* D-*values of first-order isothermal inactivation of ACP in cod muscle determined by separate linear regression analysis of short and long isothermal heat treatment*[a]

Temp (°C)	Heat-labile enzyme inactivation			Heating time for shift from D_L to D_S (s)	Enzyme inactivation after dominated by heat-stable enzyme activity			Residual ACP activity at time shift (% of A_0)
	D_L (min)	SE	R^2		D_S (min)	SE	R^2	
56	4.3	0.2	96.4	126.1	178.8	19.6	65.9	25.2
60	3.6	0.3	87.6	100.2	26.2	1.0	94.6	28.6
64	2.3	0.1	95.0	106.1	8.6	0.4	95.1	21.1
68	1.3	0.1	91.1	96.2	2.0	0.1	94.2	29.1

[a] Level of heat-stable ACP activity (as percentage of total initial enzyme activity) and time for shift in model from heat-labile to heat-stable activity is estimated by least squares of residuals of activity (*A*) predicted by equation 5.

at a higher temperature range than it was in extract, the sensitivity of the *D*-values (i.e., *z*-value) was slightly lower than that for extracts (Fig. 4). This difference might be explained by several environmental factors, e.g., reduced water content *(1, 10, 32)*.

Considerations for potential use of ACP as an intrinsic TTI. To be suitable as an intrinsic indicator, an enzyme must have identical heat sensitivity for a certain decimal reduction time (i.e., *z*-value) as the target microorganism *(30)*. For fish products, the pathogen *L. monocytogenes* is considered a target *(2)*. No stringent requirements exist with regard to the *D*-values of the TTI; however, these *D*-values should be sufficiently high in the relevant temperature domain to induce a detectable response to the heat load *(30)*.

The *z*-value of ACP in cod muscle was slightly lower than that reported for *L monocytogenes* by the European Chilled Food Federation (ECFF) *(8)*, which was 7.5°C. However, a wide range of kinetic data have been reported for *L. monocytogenes* depending on the strain and the model system used for determining the heat resistance *(4)*. For cod, Ben Embarek *(4)* investigated the heat resistance of *L. monocytogenes* O62 and O57 and found a $D_{60°C}$ of 1.7 and 2.8 min, respectively (both with a *z*-value 5.7°C). Thus, a 6-log inactivation would require a pasteurization value ($P_{60°C}$) of 10.2 to 16.8 min. The *z*-values of both strains of *L. monocytogenes* were below the *z*-value of ACP. Even

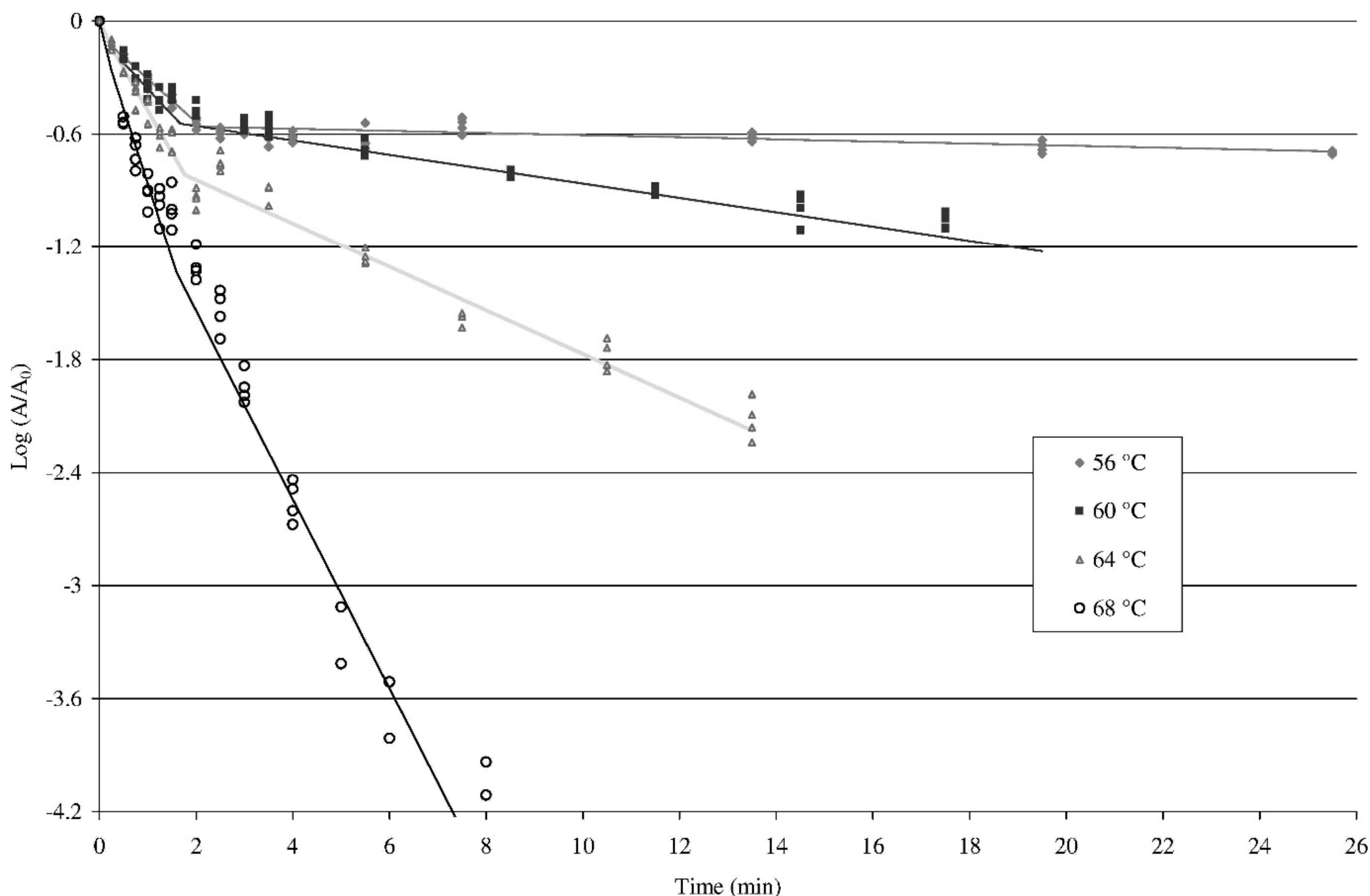

FIGURE 3. *Residual ACP activity versus isothermal heating time of cod muscle at 56°C (◆), 60°C (■), 64°C (▲), and 68°C (○) with regression lines.*

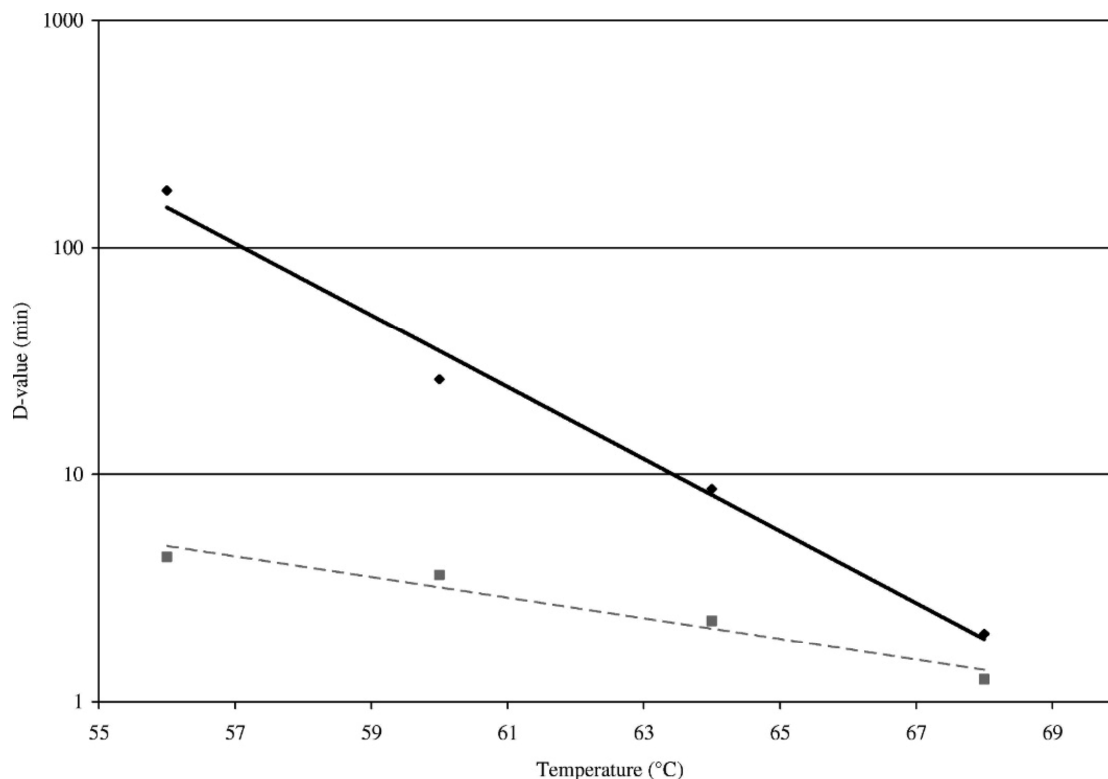

FIGURE 4. *Log* D-*values for thermal inactivation of ACP activity before time shift* (■) *and after time shift* (◆) *obtained in cooked cod muscle plotted against temperature and appurtenant regression line before time shift (dotted line,* $R^2 = 0.953$*) and after time shift (solid line,* $R^2 = 0.989$*).*

though the major kinetic requirement for use as a TTI was not met for ACP, a conservative estimate of the achieved microbiological safety level can be obtained by applying the method described by Van Loey et al. *(31)* for TTIs with a *z*-value lower than that of the target. In this method, the process value is calculated based on the response of the TTI, using a reference temperature equal to the maximum processing temperature. An equivalent time at any other reference temperature can then be estimated safely by converting the process value using the *z*-value of the target attribute.

Several other studies on *L. monocytogenes* have been published, and the maximum heat resistance found (in milk) was $D_{60^\circ C} = 16.7$ min and $z = 5.56^\circ C$ *(11)*. However, lower heat resistance of *L. monocytogenes* was reported by Doyle et al. *(7)*. A possible change in the heat resistance of *L. monocytogenes* has been suggested to occur as a result of several factors such as heat or cold shock, changes in pH and water activity (a_w), and the growth phase of the cells. Van Asselt and Zwietering *(29)* collected data ($n = 967$) for *L. monocytogenes* and found that the presence of 10% salt ($a_w < 0.92$) resulted in high resistance but other factors did not have a significant effect ($P < 0.05$) on the *D*-value. However, a 3-h cold shock to 10°C reduced the $D_{60^\circ C}$-value of *L. monocytogenes* Scott A from 1.26 ± 0.10 min to 0.69 ± 0.01 min for controls and cold shocked bacteria, respectively *(20)*. A typical industrial product consisting of vacuum-packed cod portions would not have a salt content >10% but would typically be cooled below 10°C from slaughter to processing. This cooling regimen also could be a possible explanation for the relatively low *D*-values observed by Ben Embarek *(4)* for *L. monocytogenes* in cod precooled to 2°C. Further evidence is still needed before the heat load on cod ready-to-eat processed foods with extended durability (REPFED), for which inactivation of *L. monocytogenes* is required, can be reduced to less than 2 min at 70°C or the equivalent. Thus, a heat load minimum equivalent to 2 min at 70°C is still required for cod REPFEDs where inactivation of *L. monocytogenes* is the target of the thermal processing.

The *D*-value of the enzyme was three times higher than the $D_{70^\circ C}$-value of *L. monocytogenes* of 0.33 min suggested by the ECFF *(8)*. This finding implies the possibility of using ACP for monitoring a 4-log inactivation of *L. monocytogenes*, whereas a 6-log inactivation would require a pasteurization value ($P_{70^\circ C}^{7.5^\circ C}$) of 2 min, as calculated by equation 8 (where T_c is the temperature in the cold spot of the product), or a $P_{60^\circ C}$ of 43 min.

From the regression curve for the *D*-values against temperature from 56 to 68°C, a *D*-value at 60°C of 34.93 min was calculated above for the ACP thermal inactivation in cod muscle. It is feasible to measure up to a 1.5-log reduction of enzyme activity, i.e., a $P_{60^\circ C}^{6.31^\circ C}$ value of 52.40 min can be validated, which is well above the highest values used for inactivation of *L. monocytogenes*. However, our data from in situ thermal inactivation of ACP is limited to 68°C and should not be extrapolated outside the measured range. Even if these data could be extrapolated, their use would be limited to measuring a maximum $P_{70^\circ C}$ of 1.36 min, which equals only a 4-log inactivation of the most heat-resistant *L. monocytogenes*.

Remaining ACP activity in cod heat treated at 70°C would therefore indicate that the heat load is not sufficient for a 6-log reduction of all strains of *L. monocytogenes*, but

TABLE 5. *Overview of kinetic data obtained for heat inactivation of ACP in various samples*

Sample	Temp range (°C)	$D_{60°C}$ (min)	z-value (°C)	Reference
Farmed Atlantic cod muscle	56–68	34.93 ± 2.02	6.31 ± 0.51	Current study
Farmed Atlantic cod extract	54–68 (70)	7.98 ± 1.11	6.92 ± 0.07	Current study
Farmed Atlantic cod extract with protease inhibitor	55–67.5	6.78 ± 0.10	6.37 ± 0.09	*13*
Ham			6.94	*15*
Ground beef	53–68	14.12[a]	7.41	*22*

[a] D-value was recalculated to reference temperature 60°C by using the z-value (7.41°C) and the $D_{63°C}$-value (5.56 min) published in the study cited.

ACP could possibly be used as a quantitative indicator at lower temperatures, at least with respect to some specific strains and products that are chilled before heat treatment.

In Table 5, an overview of kinetic data on heat inactivation of ACP from various samples is given. Heat-induced quality changes in cod related to heat denaturation of proteins are well known, and these data from other sources could therefore be used to compare the kinetic parameters of ACP activity to those of other proteins, if available. For actin in cod muscle, the $D_{62°C}$ is 130.1 ± 5.4 min and the z-value is 5.74 ± 0.11°C *(27)*. These values are quite different from those obtained for thermal inactivation of ACP activity in the present study, and data for other proteins of cod have not been published. Some conditionally essential nutrients, e.g., taurine (2-aminoeth-anesulphonic acid), also are significantly reduced above 63°C *(17)* and could be compared with inactivation of ACP, but unfortunately no information on heat resistance was found. ACP cannot be used to monitor loss of vitamins because their thermostability is less temperature dependent ($z > 25$°C) than that of ACP.

General discussion. ACP activity in raw cod muscle is not significantly influenced by gender. During initial cold storage at 4°C, no significant changes in ACP activity were observed from day 7 after slaughter to day 13. ACP activity is significantly reduced by freezing, but after the initial drop the activity in extracts of cod muscle ($n = 70$) was maintained at 2.54 ± 0.17 Abs/(min·g sample) during storage for 24 months at −80°C. ACP activity does not vary much during the annual season, but a significant increase was observed in June (shortly after spawning) and a minimum level of ACP activity was observed in December. Brining with different levels of salt and phosphates resulted in either a low or nonsignificant drop on the activity of ACP in cod muscle.

The thermal resistance of ACP activity in cod muscle was measured from 56 to 68°C, and the $D_{60°C}$ was 34.93 ± 2.02 min and the z-value was 6.31 ± 0.51°C (in the stable phase). This level of heat resistance is significantly higher than that of ACP when heated in extracts of cod muscle at 54 to 70°C ($D_{60°C}$ = 7.98 ± 1.11 min). The time shift from rapid ACP inactivation during the first 1 to 2 min of heating observed in situ was not obvious when evaluating heat treatment of extracts at the heating times chosen. However, the phenomenon is supposed to be similar for extracts and for the heat inactivation of ACP in situ. Some evidence for this similarity is that the regression lines used for determining D-values do not match the A_0 well for a heat treatment time of 0 min. Further investigations would require changing the methodology to obtain isothermal conditions at very short treatment times.

There was no significant difference in $D_{60°C}$ (P = 0.703) with respect to gender. The high D-value of ACP when heat treated in situ suggests that ACP could be used for monitoring a 4-log inactivation of strains of *L. monocytogenes* with the same z-value. The z-value was 6.92 ± 0.07°C for ACP activity for heat-treated extracts, a value is slightly higher than that for heat-treated muscle. However, this z-value is below the 7.5°C commonly accepted to represent *L. monocytogenes* and would lead to an overestimation of the pasteurization value for products processed at temperatures above the reference temperature. However, at temperatures below the reference temperature the pasteurization value would be underestimated. This information could be important for validating products of short shelf life. For industrial production of ready-to-eat products with an extended shelf life (>7 days), a 6-log reduction of *L. monocytogenes* is commonly required, which limits the feasibility of ACP as a TTI. However, ACP will be inactivated during a commercial pasteurization process and therefore is not a shelf life–limiting factor for heat-preserved fish products. To evaluate the use of ACP activity as an intrinsic TTI, thermal stability should be determined in the environment where the enzyme originates and not in extracts. The biphasic behavior observed should be investigated further to determine more exact time shifts and the ACP activity level at this time shift.

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Paper V

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Optimisation of heat processing of farmed Atlantic cod (*Gadus morhua*) muscle with respect to cook loss, water holding capacity, color and texture.

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Abstract

Convenience products of fish heat processed in-pack are often subjected to a compromise between quality and durability within the frames of required safety. The purpose of this study was to provide knowledge for optimising pasteurisation of cod muscle by quantifying changes in water holding capacity (WHC), cook loss, colour and texture within a wide range of processing times and temperatures.
A heat load equivalent to 2 min at 70°C may be applied to the cod while maintaining the WHC above 66 %, the cook loss below 5.6% and keeping hardness and whiteness low, provided the processing temperature is kept in the range 68°C ± 4°C.

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Introduction

Vacuum packed convenience fish products; exposed to mild heat treatment, and depending on refrigerated distribution have been available in the Norwegian retail market since 1997 (Skipnes et al., 1999). This product category, however, is challenging both with respect to microbiological safety and sensory quality, often compromising sensory attributes like tenderness and juiciness. Vacuum pacaging prior to heat processing can be favourable with respect to flavour and juiciness (Creed, 1995; Ghazala, 2004), but some water expelled from the food will be retained in the pouch and may aid the hydrolysis of collagen proteins (Ledward, 1979). Depending on raw material microbial load, storage temperature, required shelf life etc., a thermal load sufficient to inactivate a target bacterium, using a combination of process time and temperature that retain maximum quality is common practice (Skipnes et al., 2008a). Available literature on Atlantic cod (*Gadus morhua*) often considers raw material quality, while quality description of heat treated cod is less abundant.

Heating converts the translucent, jelly-like raw muscle into an opaque, slightly firm and springy form. Heat also causes damage to the connective tissue, hence, fish easily falls apart and becomes palatable on mild heating (Parry, 1970). Temperatures > 45°C causes protein denaturation (Fennema, 1990; Offer et al., 1983). Protein denaturation reduces the dimension of myofibrils and collagen, resulting in shrinkage of muscle fibre diameter and sarcomere length (Palka et al., 1999). Thus shrinkage squeezes liquid from the muscle, documented in beef (Palka et al., 1999), pork (Barbera et al., 2006) and cod and salmon muscle (Ofstad et al., 1996). The combined effect of denaturation and liquid loss is tougher texture (Fennema, 1990; Offer et al., 1983), and released liquid contains protein which may coagulate on the muscle surface as a curd.

Sarcoplasmic proteins have a very low capacity for immobilization of water in their structure (Dunajski, 1979), hence they do not contribute much to texture changes. The enzymes present in the sarcoplasmic fraction, however, may influence gelation of intact muscle.

Myosin and actin are the myofibrillar proteins directly involved in contraction/relaxation. Due to the low collagen content of fish, their role in gelation and texture is even more important than in meat (Brown, 1987). Deng (1981) observed different profiles in shear force when heating mullet to different temperatures in the range 35°C to 85°C and suggested that texture of cooked fish is tougher due to physical protein denaturation only, while a slower and/or stepwise heating process gave a tender/softer texture, explained by alkaline protease activity. Some heat stable alkaline proteases are more or less inactive at physiological temperatures and activated at high temperatures only (Dahlmann et al., 1985; Toyahara et al., 1987).

The effect of different thermal treatment on color of the cod muscle has not been documented. In sensory evaluation schemes, color ranging from white to bright yellow has been described. (Fernandez-Segovia et al., 2003).

While protein denaturation has been considered to be the main reason for both water loss and textural changes in fish during thermal processing (Ofstad et al., 1996), cook loss and water holding capacity (WHC) of cod muscle do not correlate well to the major protein transition as measured by differential scanning calorimetry (DSC) (Jensen et al., 2003; Skipnes et al., 2008b). Hence, thermodynamic mechanisms should also be reviewed. A mechanistic model could be combined with knowledge of protein denaturation. Therefore optimization of cooked fish quality may be done by modelling as discussed by Skipnes & Hendrickx (2008a). A first step on the route to build such a combined model is to measure the actual changes in the fish muscle at isothermal conditions.

The purpose of this study was to provide data for optimising pasteurisation of vacuum packed convenience cod muscle by quantifying changes in water holding capacity (WHC), cook loss, colour and texture during thermal processing within a wide range of temperatures and processing times.

Materials and methods

Raw material

The fish raw material were the same as used in our previous study (Skipnes et al., 2008b) which consisted of loins from 94 individual of farmed Atlantic cod (*Gadus morhua*) that were either separated in myotoma (for measurement of color and texture) or coarsely ground (for measurement of cook loss and WHC) in a grinder with Ø8 mm holes. In both cases the sample material was packed in polyethylene beakers (Dynopack no.567, Polimoon AS, Kristiansand, Norway) of either 125 g ground material or myotoma separated with numbered plastic sheets and stacked in layers. The beakers were stacked between layers of dry ice and stored in a freezer at -80±1ºC until further use. The main characteristics presented for the ground raw material in Skipnes et al. (2008b) was (mean ± s.d.); pH 6.22 ± 0.01, average water content about 79% (w/w), fat 0.56%, protein 20.1 %, ash 1.18 % and salt 0.105%. Further characteristics of the raw material were published by Skipnes et al. (2008b) where it was concluded that the sample material was fresh and in good microbial condition when frozen.

Heat treatment

The samples were isothermally heat treated at every 5°C in the range 20°C to 95°C in a water bath (Heto lab, Germany) for time intervals of 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40 and 60 min except for the untreated reference samples. The sample cups were immersed sideways into the water bath to avoid entrapment of air at the top or bottom lid.

Temperature abuse was carefully avoided by keeping the samples in an isolated box 0ºC between each step (filling, weighing, closing, heat treatment, centrifugation etc.) of the experiments. Filling and weighing of samples in each sample cup was done within 2-3 min in ambient room temperature ranging from 19ºC to 20ºC. The temperature of the sample, however, did not increase more than a few degrees as the fish, the sample cups and the spatula were pre-cooled to 0-2ºC.

Cook loss and WHC analysis

Cook loss and WHC were obtained by the method and associated equipment described in our previous study (Skipnes et al., 2007). The samples (n=8 or n=4) were placed in the test cell which was sealed before isothermal heating as described above. After heating, the samples were cooled and opened for removal of the cook loss which was determined by weighing cup and sample before and after removal of cook loss. The cups were sealed with a bottom lid for collection of exudates and centrifuged (Hettich GmbH & Co, Tuttlingen, Germany) at 528 * G for 15 min at 4°C. WHC was determined as the total weight of cook loss and liquid expelled during centrifugation in percent of the initial water content of the fish. Dry matter content of fish muscle was determined gravimetrically after drying at 105ºC for 16 hours and used for calculation of WHC for the fish samples.

Determination of color and texture

The color and texture measurements were performed on myotoma cut from the cod loins with a thin, sharp blade and stored at -80°C. The myotoma were thawed and placed in the sample cups and heat treated and cooled as previously described, except for raw reference samples. Colour was measured with a colorimeter (Minolta Croma Meter CR-300, Minolta Co. LTD, Osaka, Japan) with the probe fit into the sample cup for direct measurement (in triplicate) before texture measurements. Whiteness, W, was determined with equation 1 as demonstrated by Park (1994).

$$W = 100 - \sqrt{(100 - L^{*})^{2} + a^{*2} + b^{*2}} \qquad (1)$$

where L^* is lightness (0 to 100%), a^* color ranging from green to red and b^* color range from yellow to blue. The advantage of using whiteness is a better resolution for the relatively small difference between white to slightly yellowish. Whiteness has been used as parameter in several studies on surimi and on raw or dried cod (Benjakul et al., 2004; Skjerdal, 2002; Tabilo-Munizaga et al., 2004; Uresti et al., 2003)

The texture analyses were performed with a Texture Analyser TA.XTplus (Stable Micro Systems Ltd, UK), equipped with a 5 kg load cell. A TPA (Texture Profile Analysis) test was conducted on each dissected myotome while situated in the sample cup. The TPA

test consists of compressing a small piece of food two times in a way that imitates the action of the jaw and extracting from the resulting force-time curve a number of textural parameters that has been found to correlate well with sensory evaluation (Bourne, 1978). The primary parameters of hardness, cohesiveness, springiness (elasticity), and adhesiveness, and the secondary (or derived) parameters of fracturability (brittleness), chewiness and gumminess can be determined by the TPA (Szczesniac, 1966) and the hardness was analysed in the present study. The myotome samples were measured using a flat ended cylinder of Ø½" of type P/0.5 AOAC for gelatine (Stable Micro Systems Ltd., UK). The trigger force for measuring height of the sample was 10,0 g and the cylinder was moving at 1,0 mm/sec until 80% compression of the sample. All samples were stored on ice prior to analysis. Thus, the temperature of the samples was 0-1ºC.

Experimental design and statistical analysis

The design of the experiment was created in, and the results statistically analysed using Minitab R14.13 (Minitab Inc, USA). Color and texture was analysed by the one-way Anova in Minitab, while WHC and cook loss were determined by full factorial designs.

Results and discussion

Cook loss

The moisture content of raw, ground cod muscle (n=90) as measured in a drying chamber at 105ºC for 16 hours was aproximately 78 %. The moisture content of all the cooked, ground cod samples (pooled over all temperatures, n = 158) was aproximately 78 %as well. There was a significant cook loss during heat treatment as shown in figure 1, which could be explained by a dry matter content of the expelled moisture close to the dry matter content of the fish. This is slightly different from findings in salmon heat treated at temperatures > 100°C where the dry matter content of the cook loss was < 15% (Kong et al., 2007a). The results in the present study was obtained by separating the cook loss from the fish while the fish was still hot and may be different compared to other methods for cook loss determination that separates the expelled moisture from the fish after cooling. If the moisture is cooled while it is still surrounding the fish (e.g. in a vacuum pouch), the dissolved proteins will coagulate on the fish surface which means it will not drip off as long as the fish is refrigerated.

Analysed as a two factorial design by Anova, the effects of both time and temperature as well as the interaction of time and temperature was highly significant (P<0.0001). However, some local extremes shown in figure 1 for the time and temperature combinations 25°C/10 min, 40°C/10 min, 50°C/45 min, 60°C/5, 60°C/30 min, 75°C/10 min and 80°C/45 min were not significant different (P>0.05) from the nearest time/temperature combinations measured.

The cook loss was increased with increasing temperature which is also the trend seen in other studies (Kong et al., 2007a; Ofstad et al., 1993). Even if these studies cannot be directly compared to the present study, they all confirm the trend of increasing cook loss with increasing time and temperature. Skipnes et al. (2007) showed that the cook loss was moderate during heating in the range 40ºC to 70ºC but increased rapidly up to 100ºC. A similar trend was seen by Skipnes et al. (2008b), but the major increase in cook loss was shown to be at a temperature above 80ºC, i.e. in a temperature range where the major protein denaturation was completed and changes in cook loss were small (average cook loss < 4 %).

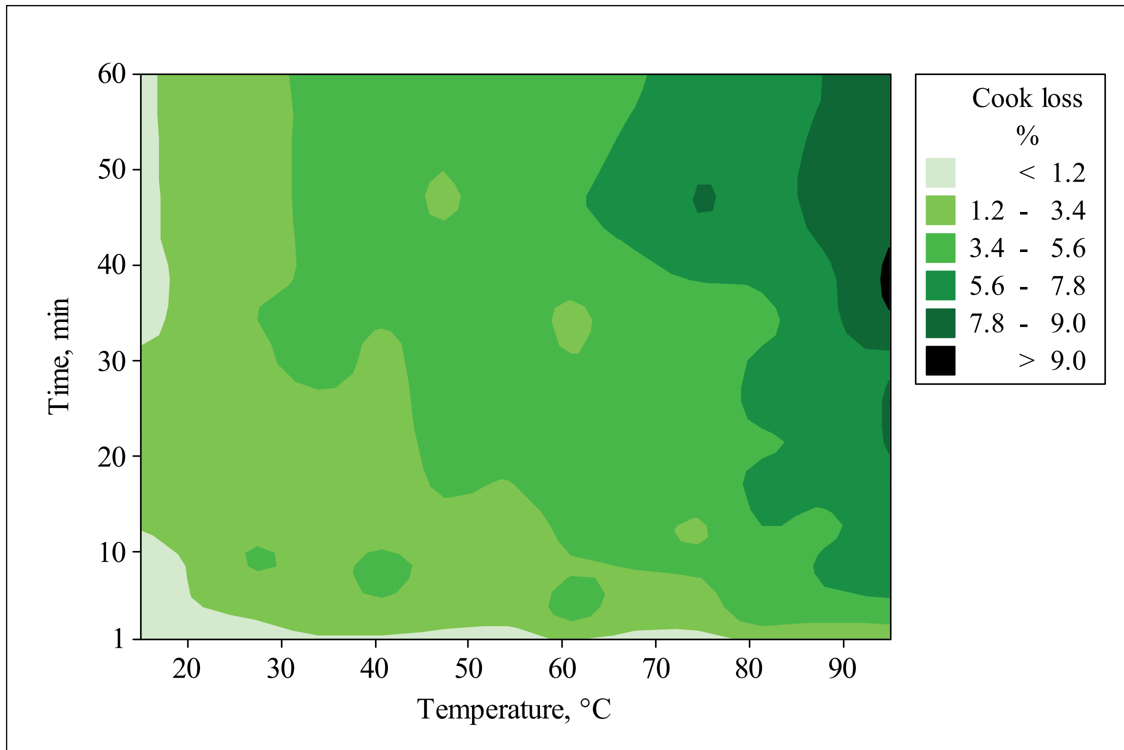

Figure 1. Cook loss in % of mass of raw fish as function of time at temperatures ranging from 20°C to 95°C.

For optimization of mild heat treatment this is of interest as it opens up the possibility of a process with denatured proteins and thus a product with cooked appearance, while the cook loss is still moderate. In the same temperature region it will also be possible to adequately inactivate *Listeria monocytogenes* (70°C for 2 min). The shelf life will however be limited as long as other pathogens, i.e. non-proteolytic *Clostridium botulinum* type E, and their spores are not inactivated. The cook loss increased rapidly during the first few minutes and continued to increase more moderately at longer heat treatment times.

The primary mechanism leading to cook loss is thermal denaturation of muscle proteins (Bell et al., 2001) and heating of cod muscle has been observed to cause denaturation of myosin and shrinkage of myofibrils which leads to expulsion of water (Ofstad et al., 1993). However, the cook loss continued to increase when the samples were heated to higher temperatures than required for the proteins to denaturate and the water in the sample could be expected to be available for expulsion by a weak mechanical force. Such a

mechanical force could be due to gradients of temperature, pressure and water concentration and will obviously lead to expulsion of water from any porous media that is heated, provided it contains free water. Fish, like most foods, have small pores or capillary pores that may contain water. Most of the water is located within the myofibrils, in the narrow channels between thick and thin filaments (Bertola et al., 1994; Offer et al., 1983). There are primarily three transport mechanisms for fluids in food; molecular diffusion (for gases), capillary diffusion (for liquids) and convection (pressure driven or Darcy flow). A mechanistic model of this fluid transport has been described by Ni, Datta and Torrance (1999) and has been presented as equations for total flux of vapour and liquid driven by;

(1) a pressure gradient
(2) a gradient of water concentration
(3) a temperature gradient

in the food.

The observed cook loss can be discussed in light of such a mechanistic model:

(1) Provided the density, permeability and viscosity of water is constant (true under isothermal conditions), the water flux is increasing linearly with increasing pressure. However, the pressure gradient is not increasing linearly since it is a result of
    (a) muscle shrinkage caused by several steps of thermal protein denaturation, i.e. a function of temperature and time
    (b) thermal expansion of water and gases in the fish meat, i.e. a function of temperature
(2) Water expelled will surround the fish and a concentration gradient will build up and contradict the expulsion of water. Depending on the time/temperature history and the condition of the fish, proteins will be dissolved in the expelled water and decrease the concentration gradient. Water will also be released from proteins and increase the concentration gradient in the opposite direction.
(3) The temperature gradient is given by the heat equation and will be log-linearly decreasing from a maximum when heating is started and approach zero as the temperature in the fish is evening out. This is consistent with the observation of little or no increase in cook loss over time at constant temperature after the protein

denaturation, which is completed within a few minutes at constant temperature.

The three mechanisms discussed above are all influenced by the protein denaturation. It has previously been shown that heat induced structural changes (Ofstad et al., 1993) and protein denaturation (Skipnes et al., 2008b) is irreversible. During cooling the new configuration of the fish meat established during heating will remain unchanged. Only a small amount of the liquid expelled during heating will be absorbed by the fish during cooling and it is not expected to cause any swelling but rather follow a mechanistic model. Further research on protein denaturation would indeed help in establishing this model, but it is possible this could be studied indirectly by measuring shrinkage of fish meat during heating. Studies of salmon heated at temperatures in the range 100°C to 130°C has shown that the cook loss is relatively constant after 30 min heating time and this plateau level was a function of the temperature and correlated well with area shrinkage of a disk shaped sample (Kong et al., 2007b). This rapid increase before reaching the plateau level seems to be the same behaviour as seen from the cod at temperatures below 100°C.

WHC during isothermal heating

The results for WHC screened by isothermal heating for 1 to 60 min at temperatures in the range of 20ºC to 95ºC are shown in figure 2. Long processing times and high temperatures resulted in a minimum WHC. When heating at temperatures 20°C and 25°C the reduction in WHC is not statistically significant (P=0.19 and P=0.95 respectively) from raw samples. At 30°C the WHC is maintained at the initial level the first 5 min but then decreases with increasing heating time and is significantly lower than raw samples (P<0.0001). At temperatures from 35°C to 95°C the WHC decreases very rapidly and reaches a temporary minimum within 2 to 30 min depending on temperature. This local minimum of WHC is in general reached faster at high temperatures (i.e. above 70°C) compared to lower temperatures.
Decreasing WHC with increasing temperature is in line with previous observations at 10 min heating time where cod muscle heated to temperatures in the range 40°C to 95°C showed a temporarily increase

in WHC after reaching the local minimum (Skipnes et al., 2008b). As discussed for cook loss, there is a loss in WHC for every group of proteins that are denaturated as demonstrated by DSC measurements (Skipnes et al., 2008b).

For the purpose of preparing a meal, it can be expected that the heat load applied to the product always exceeds a specific heat load, e.g. 40°C and 5 min for a product to be served immediately after heat treatment. Above this heat load there is a local maximum for WHC shown in fig 2 (medium dark green area), ranging from approximately 55°C to 75°C and from approximately 20 min to 30 min. This is consistent with earlier findings (Ofstad et al., 1993; Skipnes et al., 2008b). This finding is interesting for optimizing the thermal process with respect to WHC as it would be possible to achieve a 6 log inactivation of *Listeria monocytogenes* within this heating range (Skipnes et al., 2010) and maintain a relatively high WHC.

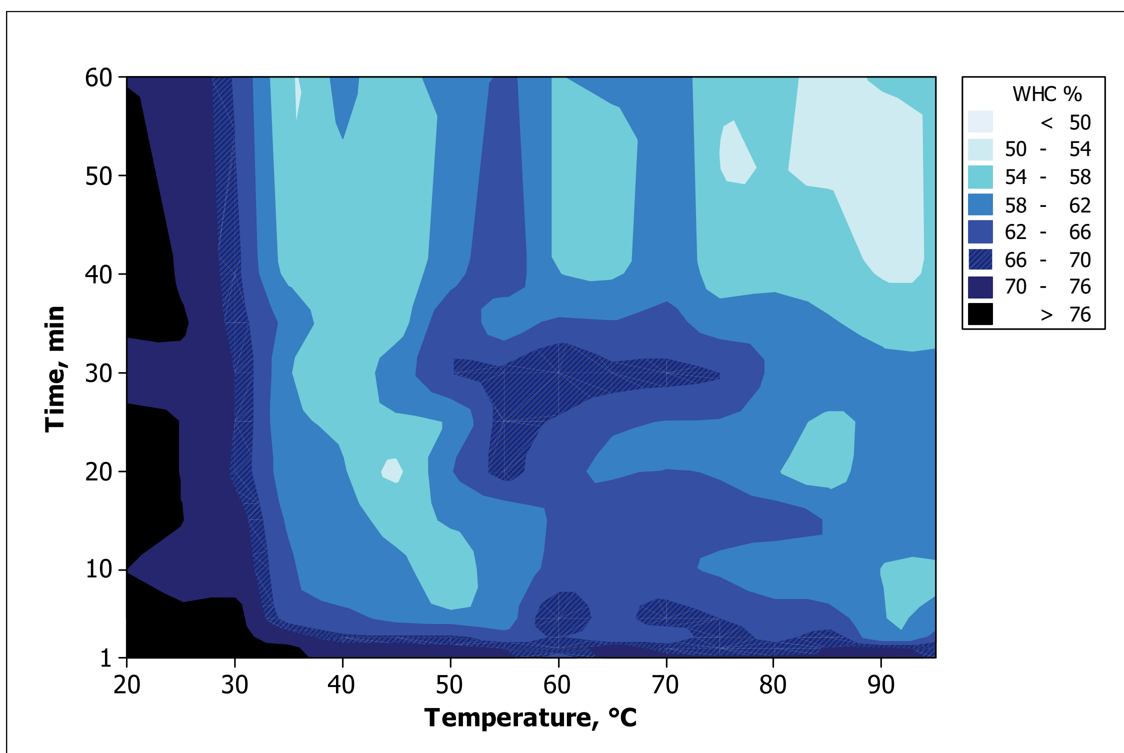

Figure 2 Water holding capacity (WHC) at cooking times from 1 min to 60 min and heat treatment temperature from 20ºC to 95°C.

Texture.

Mean hardness of raw myotoma of cod loin with standard error was 340±80 g. Heat processing resulted in a harder fish muscle as shown in table 1. The minimum hardness was found at low temperatures (at 50°C and 40°C when processing for 10 and 40 min respectively). Maximum hardness was found at the maximum temperature of 80°C. However, the hardness of samples heat treated at different temperatures is not significantly different from each other. Neither was there any difference between samples processed for 10 min and 40 min regardless of processing temperature. For samples heat processed at 80°C there was no significant increase in hardness when comparing results from 40 min to 10 min processing time. It has previously been shown that texture is closely related to the water content of the muscle (Dunajski, 1979) and a correlation between WHC and texture may therefore also be expected. This could explain the softer texture observed for cod muscle heat treated for 10 min at 70°C compared to 10 min at 60°C and 90°C.

Table 1. Mean hardness of myotoma of cooked cod loin in g and standard error.

Time, min	Temperature °C				
	40	50	60	70	80
10	850± 100	590±120	910±150	620±50	1120±320
40	620±100	680±110	1000±150	1090±270	1150±280

Color.

When heated, the opaque cod muscle changes to white. Raw fish had a whiteness of 52. 35 ± 6.43 and heat treatment for 10 min at 30°C does not result in any visible changes or measurable changes in terms of whiteness. The mean whiteness observed for the heat treated cod is shown in figure 3. Processing times in the range 5 to 40 min did not change the whiteness score within each temperature tested. However, heating at 40°C for 10 or 40 min resulted in a much higher whiteness score than heating at 30°C. The whiteness after heating at 70°C, 80°C and 90°C was not significantly different from each other and were all higher than for temperatures below 60°C. The color changes between 30°C and 40°C coincidences with the changes in WHC and the denaturation of myosine and the continuing increase in whiteness at increasing temperatures coincidences with the protein denaturation.

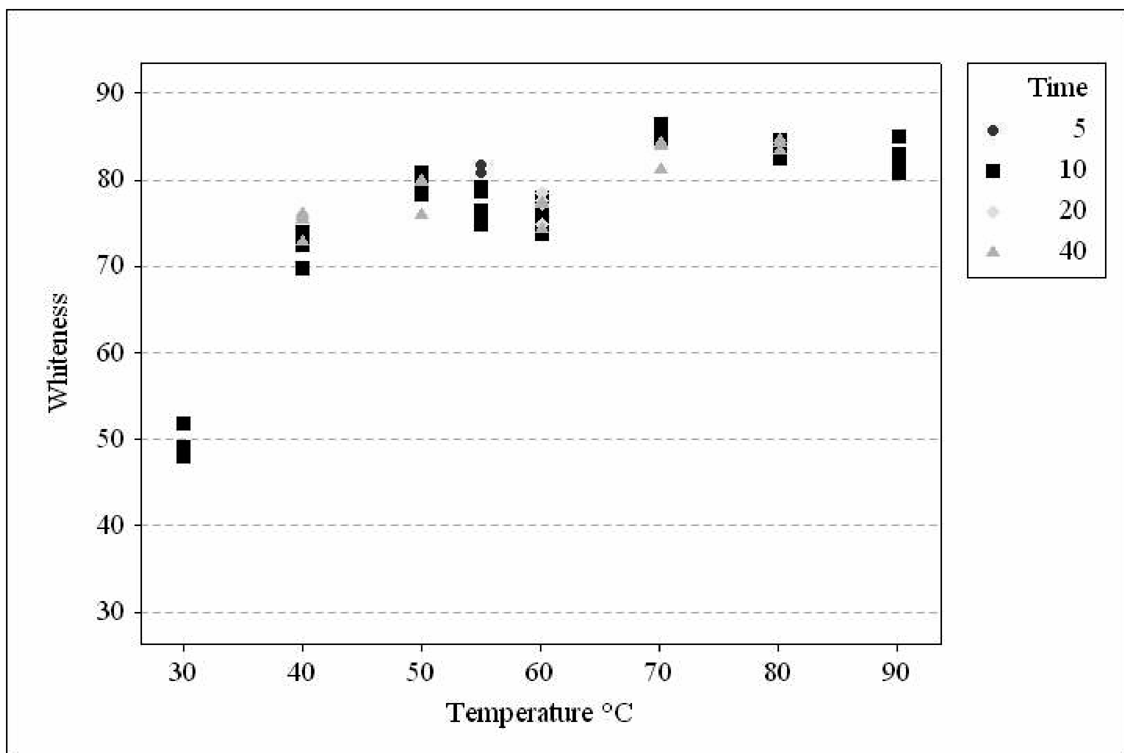

Figure 3. Mean whiteness of cod cooked for times ranging from 5 to 40 min.

Conclusion

Cook loss and reduction in WHC as a result of heat treatment of muscle from farmed Atlantic cod has been quantified. It has been shown that the reduction in water holding capacity occurs as soon as the temperature is high enough to initiate protein denaturation (i.e. T > ~30°C) and the decrease is very pronounced during the first few minutes. After ~10 min the reduction is less pronounced and the WHC even increase until it continues to decrease at a nearly constant rate. This results in a local maximum of WHC which coincidences with the minimum heat load required for a 6 log inactivation of *Listeria monocytogenes* and is interesting with respect to optimizing the thermal process. A process aiming for inactivation of *Listeria monocytogenes* may be achieved while maintaining the WHC above 66 % and the cook loss below 5.6% and keeping hardness and whiteness below the maximum values found in the study, as long as the processing temperature is kept in the approximate range 68°C ± 4°C. The processing time should also be limited in the approximate range 25 to 35 min. Whiteness and hardness is increasing with increasing temperatures.

The cook loss and changes in WHC has been expected to be a result of protein denaturation by several authors, but in the present study it is shown that protein denaturation is only one of the parameters affecting WHC and cook loss. In fact, the WHC continued to decrease and the cook loss increased when the temperature was increased above 70°C and up to 95°C, i.e. in a range where no protein denaturation is observed by DSC. The cook loss observed in this temperature range follow a pattern that possibly could be described by a mechanistic model, but at temperatures below 70°C protein denaturation would have to be implemented in the terms for pressure and concentration gradients to explain the initial drop that appears as soon as the proteins start to denaturate and releases water bound to the proteins.

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